

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>1038-1183 MIS:jb</b>
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR <b>09/914622</b>
INTERNATIONAL APPLICATION NO. <b>PCT/CA00/00207</b>	INTERNATIONAL FILING DATE <b>February 29, 2000</b>	PRIORITY DATE CLAIMED <b>March 3, 1999</b>
TITLE OF INVENTION <b>MULTI-COMPONENT VACCINE COMPRISING AT LEAST THREE ANTIGENS TO PROTECT AGAINST DISEASE CAUSED BY HAEMOPHILUS INFLUENZAE</b>		
APPLICANT(S) FOR DO/EO/US <b>Sheena M. Loosmore; Yan-Ping Yang; and Michel H. Klein</b>		
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2))</p> <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ul> <p><input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto.</li> <li>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</li> </ul> <p><input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))</p> <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input type="checkbox"/> have been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ul> <p><input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p><input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). - <b>unsigned copy</b></p> <p><input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).</p> <p>11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409).</p> <p>12. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210).</p> <p><b>Items 13 to 20 below concern document(s) or information included:</b></p> <p>13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>15. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p>16. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>17. <input type="checkbox"/> A substitute specification.</p> <p>18. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>19. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>20. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>21. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>22. <input type="checkbox"/> Certificate of Mailing by Express Mail</p> <p>23. <input checked="" type="checkbox"/> Other items or information:</p>		
<p><b>Initial Information Data Sheet</b></p> <p style="text-align: right;">O I P E J S T S E C P A T E N T &amp; T R A D E M A R K O F F I C E</p> <p style="text-align: right;">AUG 31 2001</p>		

ON NO. (IF KNOWN, SEE 37 CFR  
09/914622INTERNATIONAL APPLICATION NO.  
PCT/CA00/00207ATTORNEY'S DOCKET NUMBER  
1038-1183 MIS:jb

The following fees are submitted:.

**CALCULATIONS PTO USE ONLY****SIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .....	\$1000.00
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO .....	\$860.00
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....	\$710.00
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) .....	\$690.00
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) .....	\$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =****\$860.00**Surcharge of \$130.00 for furnishing the oath or declaration later than  20  30 months from the earliest claimed priority date (37 CFR 1.492 (e)).**\$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	27 - 20 =	7	x \$18.00	<b>\$126.00</b>
Independent claims	2 - 3 =	0	x \$80.00	<b>\$0.00</b>
Multiple Dependent Claims (check if applicable).			<input checked="" type="checkbox"/>	<b>\$270.00</b>

**TOTAL OF ABOVE CALCULATIONS =****\$1,256.00**

<input type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	\$0.00
<b>SUBTOTAL =</b>	<b>\$1,256.00</b>

Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).	<input type="checkbox"/> +	\$0.00
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**TOTAL NATIONAL FEE =****\$1,256.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).	<input type="checkbox"/>	\$0.00
<b>TOTAL FEES ENCLOSED =</b>	<b>\$1,256.00</b>	

<input type="checkbox"/> Amount to be: refunded	\$
<input type="checkbox"/> charged	\$

a.  A check in the amount of \$1256.00 to cover the above fees is enclosed.

b.  Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c.  The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 192253 A duplicate copy of this sheet is enclosed.

d.  Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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24223

PATENT TRADEMARK OFFICE

SIGNATURE

Michael I. Stewart

NAME

24,973

REGISTRATION NUMBER

August 30, 2001

DATE

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re National Phase of International

Appl'n. No. : PCT/CA00/00207

Filed : February 29, 2000

Applicant : Sheena M. Loosmore, et al.

Title : MULTI-COMPONENT VACCINE COMPRISING AT LEAST  
THREE ANTIGENS TO PROTECT AGAINST DISEASE  
CAUSED BY HAEMOPHILUS INFLUENZAE

Docket No. : 1038-1183 MIS:jb

August 30, 2001

**BY COURIER**

The Commissioner of Patents  
and Trademarks,  
Washington, D.C. 20231,  
U.S.A.

**PRELIMINARY MENDMENT**

Sir:

Please amend the above-identified application as follows:

**In the Specification:**

Before the first line of the specification, add the following:

**" REFERENCE TO RELATED APPLICATIONS**

This application is a national phase application under 35 U.S.C. 371 of  
PCT/CA00/00207."

**REMARKS/ARGUMENTS**

The specification has been amended on page 1 to reflect that this application  
is a U.S. National Phase filing under 35 U.S.C. 371 of PCT/CA00/00207.

Attached hereto is a marked-up version of the changes made to the  
specification by the current amendment. The attached page is captioned "Version with  
markings to show changes made."

Respectfully submitted,

SIM & McBURNEY



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Appl. No.

**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In the Specification:**

Before the first line of the specification, add the following:

**"REFERENCE TO RELATED APPLICATIONS"**

This application is a national phase application under 35 U.S.C. 371 of  
PCT/CA00/00207."

TITLE OF INVENTION

**MULTI-COMPONENT VACCINE COMPRISING AT LEAST  
THREE ANTIGENS TO PROTECT AGAINST DISEASE  
CAUSED BY HAEMOPHILUS INFLUENZAE**

5

FIELD OF INVENTION

The present invention relates to the field of vaccinology and, in particular, to a multi-component vaccine comprising recombinant proteins from *Haemophilus influenzae* which is useful in protecting against disease caused by *Haemophilus influenzae* including otitis media.

10

BACKGROUND OF THE INVENTION

*Haemophilus influenzae* is the cause of several serious human diseases such as meningitis, epiglottitis, septicemia and otitis media. There are six serotypes of *H. influenzae*, designated a to f, that are identified by their capsular polysaccharide. *H. influenzae* type b (Hib) was a major cause of bacterial meningitis until the introduction of several Hib conjugate vaccines in the 1980's (ref. 1, throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains Full bibliographic information for each citation is found at the end of the specification immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). Vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid (ref. 2), tetanus toxoid (ref. 3 and US patent 4,496,538), or *Neisseria meningitidis* outer membrane protein (ref. 4) have been effective in reducing *H. influenzae* type b-induced meningitis. The other serotypes of *H. influenzae* are associated with invasive disease at low frequencies, although there appears to be an increase in the incidence in disease caused by these strains as the incidence of Hib disease declines (refs. 5 and 6). Non-encapsulated or nontypeable *H. influenzae* (NTHi) are also responsible for a wide range of human diseases including otitis media, epiglottitis, pneumonia and tracheobronchitis. The incidence of NTHi-induced disease has not been affected by the introduction of the Hib vaccines (ref. 7).

Otitis media is the most common illness of early childhood, with 60 to 70% of all children, of less than 2 years of age, experiencing between one and three ear infections (ref. 8). Chronic otitis media is responsible for hearing, speech and cognitive impairments in children. *H. influenzae* infections account for about 5 30% of the cases of acute otitis media and about 60% of chronic otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. It is estimated that an additional \$30 billion is spent per annum on adjunct therapies, such as speech 10 therapy and special education classes. Furthermore, many of the causative organisms of otitis media are becoming resistant to antibiotic treatment. An effective prophylactic vaccine against otitis media is thus desirable.

During natural infection, surface-exposed outer membrane proteins that stimulate an antibody response are potentially important targets for bactericidal 15 and/or protective antibodies and therefore potential vaccine candidates. Barenkamp and Bodor (ref. 9) demonstrated that convalescent sera from children suffering from otitis media due to NTHi, contained antibodies to high molecular weight (HMW) proteins. About 70 to 75% of NTHi strains express the HMW proteins and most of these strains contain two gene clusters termed *hmw1ABC* and 20 *hmw2ABC* (refs. 10, 11). The HMWA proteins have been demonstrated to be adhesins mediating attachment to human epithelial cells (ref. 12). Immunization with a mixture of native HMW1A and HMW2A proteins resulted in partial protection in the chinchilla intrabulla challenge model of otitis media (ref. 13)

US Patent No. 5,603,938 (Barenkamp), assigned to St. Louis University 25 and Washington University and the disclosure of which is incorporated herein by reference, describes the cloning, expression and sequencing of the genes encoding the HMW1 and HMW2 proteins from strain 12 of non-typeable *Haemophilus influenzae*. The HMW proteins are a family of proteins from non-typeable *Haemophilus influenzae* of molecular weight of about 120 to 125 kDa which are 30 absent from encapsulated strains of *Haemophilus influenzae*.

The production of native HMW proteins from *H. influenzae* strains is very low and a method for producing protective recombinant HMW (rHMW) proteins

has been described in copending United States Patent Application No. 09/167,568 filed October 7, 1998 (WO 00/20609), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. A chinchilla nasopharyngeal colonization model has been developed specifically to demonstrate vaccine efficacy of adhesins (ref. 14) and the rHMW proteins are protective in this model as described in the aforementioned copending US Patent Application No. 09/167,568 (WO 00/20609). The rHMW1A and rHMW2A proteins were shown to afford equivalent protection and the rHMW1A protein was chosen for further vaccine studies. In this application, rHMW refers to recombinant HMW1A from NTHi strain 12, although other corresponding recombinant HMW1A proteins from other NTHi strains and corresponding HMW2A proteins from NTHi strains may be employed. The corresponding naturally-occurring proteins may be employed.

A second family of high molecular weight adhesion proteins has been identified in about 25% of NTHi and in encapsulated *H. influenzae* strains (refs. 15, 16, 17). The NTHi member of this second family is termed *Haemophilus influenzae* adhesin or Hia and the homologous protein found in encapsulated strains is termed *Haemophilus influenzae* surface fibril protein or Hsf.

U.S. Patent No. 5,646,259 (St. Geme, III et al), assigned to St. Louis University and Washington University, and the disclosure of which is incorporated herein by reference, describes the cloning, expression and sequences of genes encoding the Hia and Hsf proteins, which have limited homology to the HMW1 and HMW2 proteins of USP 5,603,938.

The *hia* gene was originally cloned from an expression library using convalescent sera from an otitis media patient, which indicates that it is an important immunogen during disease. The prototype Hia and Hsf proteins demonstrate about 82% sequence similarity, although the Hsf protein is considerably larger. The proteins are comprised of conserved amino and carboxy termini and several repeat motifs, with Hsf containing more repeat sequences than Hia.

United States Patent Application No. 09/268,347 filed March 16, 1999 (WO 00/55191), assigned to the assignee hereof and the disclosure of which is

incorporated herein by reference, describes the production of full-length and N-terminal truncated versions of the Hia protein (rHia) in *E. coli*. These recombinant proteins have been demonstrated to protect against bacteremia caused by *H. influenzae* type a and type b organisms, and to confer partial protection 5 against nasopharyngeal colonization by non-typeable *H. influenzae*. In this application, rHia refers to V38 rHia from NTHi strain 11, although other recombinant full-length and N-terminal truncated His proteins from other NTHi strains may be employed. The corresponding naturally-occurring proteins may be employed.

10 When under environmental stress, such as high temperature, organisms overproduce stress response or heat shock proteins (hsps). Bacterial hsps have been shown to be important immunogens, stimulating both B cells and T cells (ref. 18). The bacterial HtrA or DegP heat shock proteins are expressed under conditions of stress and the *H. influenzae* HtrA or Hin47 protein has been shown 15 to be a partially protective antigen in the intrabulla challenge model of otitis media (ref. 19). The HtrA proteins are serine proteases and their proteolytic activity makes them unstable. In addition, as components of a multicomponent vaccine, the wild-type HtrA protein will degrade mixed antigens. The site-directed mutagenesis of the *H. influenzae* *htrA* gene (termed *hin47*) and the properties of 20 the mutants have been fully described in U.S. Patent No. 5,506,139 (Loosmore et al), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference.

US Patent No. 5,506,139 (Loosmore et al) describes the preparation of analogs of *Haemophilus influenzae* Hin47 protein which have a decreased 25 protease activity which is less than about 10% of that of the natural Hin47 protein and which preferably have substantially the same immunogenic properties as natural Hin47 protein. The patent also describes the isolation, purification and characterization of nucleic acid molecules encoding the Hin47 analogs. The natural Hin47 protein is immunologically conserved among non-typeable and type 30 b isolates of *H. influenzae*. The amino acid sequence of the natural Hin47 protein and the nucleotide sequence of the encoding *hin47* gene are described in WO 94/00149 published January 6, 1994 and incorporated herein by reference.

4a

The Hin47 analogs of US Patent No. 5,506,139 are prepared by deleting or replacing by a different amino acid at least one amino acid of the natural Hin47

contributing to protease activity or by inserting at least one amino acid into the natural Hin47 protein, as specifically described therein. The at least one deleted or replaced amino acid may be selected from amino acids 195 to 201 of Hin47 and specifically may be Serine-197, which may be deleted or replaced by alanine. In 5 addition, the at least one deleted or replaced amino acid may be His-91 and may be deleted or replaced by alanine, lysine or arginine. Furthermore the at least one deleted or replaced amino acid may be Asp-121 and may be deleted or replaced by alanine.

In copending United States Patent Application No. 08/487,167 filed June 10 7, 1995, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference (WO 96/03506), there are described multiple mutations effected at different amino acids of the natural Hin47 protein to provide the non-proteolytic Hin47 analog.

In the present invention, the mutation of histidine 91 to alanine (sometimes 15 termed herein "H91A") is employed as illustration of the mutant Hin47 protein, although other Hin47 mutants with reduced protease activity as described in the aforementioned patent and application may be used.

The non-proteolytic HtrA analogue H91A Hin47, has been shown to be a protective antigen against bacteremia caused by *H. influenzae* type b and against 20 otitis media caused by non-typeable *H. influenzae* (ref. 19). HtrA was found in all strains examined, including all encapsulated strains of *H. influenzae*.

The main goal of a prophylactic vaccine against otitis media is to prevent the establishment of nasopharyngeal colonization by including adhesins as immunogens. The *H. influenzae* HMW and Hia proteins are adhesins that have 25 been shown to prevent colonization. However, since there may be a small percentage of *H. influenzae* strains that do not contain the *hmw* or *hia* genes and a wide spectrum of disease protection is desired, the H91A Hin47 antigen has been added to provide protection against such strains, although any other non-proteolytic analogs of Hin47 may be employed. The present invention provides a 30 multi-component vaccine to protect against colonization and disease caused by encapsulated or non-encapsulated *H. influenzae* organisms.

It would be desirable to provide efficacious combination vaccines comprising *H. influenzae* components containing selected relative amounts of selected antigens.

#### SUMMARY OF THE INVENTION

5 The present invention is directed towards the provision of a multi-component vaccine comprising at least three antigens, to protect against disease caused by infection with *Haemophilus influenzae*, including otitis media.

In accordance with one aspect of the present invention, there is provided an immunogenic composition for conferring protection in a host against disease 10 caused by infection by *Haemophilus influenzae*, including otitis media, comprising at least three different antigens of *Haemophilus influenzae*, at least two of which different antigens are adhesins.

One of the antigens which is an adhesin may be a high molecular weight 15 protein (HMW) of a non-typeable strain of *Haemophilus*, particularly an HMW1 or HMW2 protein of the non-typeable strain, which may be produced recombinantly.

The other of the antigens which is an adhesin is a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae* or a *Haemophilus influenzae* surface fibril (Hsf) protein of a typeable strain of 20 *Haemophilus influenzae*.

The antigen of *Haemophilus influenzae* which is not an adhesin may be a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae*. The non-proteolytic heat shock protein of a strain of *Haemophilus influenzae* may be an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease 25 activity which is less than about 10% of that of the natural Hin47 protein.

In accordance with a preferred embodiment of the aspect of the invention, there is provided an immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae*, including otitis media, which comprises:

30 (a) an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of natural Hin47 protein,

(b) a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae*, and

(c) a high molecular weight (HMW) protein of a strain of non-typeable *Haemophilus influenzae*.

5 The analog of Hin47 protein may be one in which at least one amino acid of the natural Hin47 protein contributing to protease activity has been deleted or replaced by a different amino acid and which has substantially the same immunogenic properties as natural Hin47 protein.

10 Such at least one amino acid may be selected from the group consisting of amino acids 91, 121 and 195 to 207 of natural Hin47 protein. Specific mutants which may be used including serine-197 replaced by alanine, Histidine-91 replaced by alanine, lysine or arginine and Asp-121 replaced by alanine.

15 The HMW protein of the non-typeable strain of *Haemophilus influenzae* may be a HMW1 or HMW2 protein and may be recombinantly produced. The HMW1 and HMW2 proteins are derived from the respective strains of non-typeable *Haemophilus influenzae* and possess respective molecular weights as set forth in the following Table I:

**TABLE I**

	Non-typeable <i>H. influenzae</i> Strain					
	12	JoyC	K21	LCDC2	PMH1	15
Mature Protein: HMW1	125	125.9	104.4	114.0	102.4	103.5
HMW2	120	100.9		111.7	103.9	121.9

The Hia protein may be produced recombinantly and may comprise the N-terminal truncated V38 rHia protein.

20 The immunogenic composition of the invention may be further formulated with an adjuvant. Such adjuvant for use in the present invention may include (but not limited to) aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, ISCOM matrix, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octadecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, ISCOPREP, DC-chol, DDBA and a lipoprotein and other adjuvants. Advantageous combinations of adjuvants are described in copending United States Patent Applications No. 08/261,194 filed

June 16, 1994 and 08/483,856 filed June 7, 1995, assigned to the assignee hereof and the disclosures of which are incorporated herein by reference (WO 95/34308, published November 21, 1995). The adjuvant preferably may comprise aluminum phosphate or aluminum hydroxide (collectively known as alum).

5 The components of the immunogenic composition may be present in appropriate quantities to provide the desired immune response. The components may be formulated as a vaccine for *in vivo* administration to the host. The vaccine composition may contain:

- (a) about 25 to 100 µg of the Hin47 protein,
- 10 (b) about 25 to 100 µg of the Hia protein, and
- (c) about 25 to 100 µg of the HMW protein.

The immunogenic compositions may be formulated with other antigenic components to provide a multivalent vaccine in which the additional antigenic component(s) confer protection against disease caused by another pathogen(s).

15 Such additional antigens should be such that and should be present in quantities such that the immunogenicity of the individual components of the resulting vaccine is not impaired by other individual components of the composition. Such additional antigens preferably are purified antigens in defined quantities to provide a component vaccine.

20 Such additional antigens may be those traditionally found in multivalent protective vaccines, such as diphtheria toxoid, tetanus toxoid and pertussis antigens, including pertussis toxoid, filamentous hemagglutinin, pertactin and/or agglutinogens.

25 The resulting multivalent vaccine also may contain non-virulent poliovirus, such as inactivated poliovirus, which may be type 1, type 2 and/or type 3 poliovirus. The multi-component vaccine further may comprise a conjugate of a tetanus or diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae*, preferably PRP-T.

30 The invention extends to a method of immunizing a host against disease caused by infection by *Haemophilus influenzae*, including otitis media, which comprises administering to the host an immunoeffective amount of the immunogenic composition provided herein.

Advantages of the present invention include a multi-component vaccine that can confer protection against encapsulated and non-encapsulated *Haemophilus influenzae* diseases in a safe and efficacious manner.

#### BRIEF DESCRIPTION OF DRAWINGS

5 The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 contains bar graphs A and B, illustrating the anti-H91A Hin47 immune responses for H91A Hin47 + rHMW + rHia combination vaccines in mice. Panel A, 0.3 µg each of H91A Hin47 + rHMW and increasing amounts of 10 rHia. Panel B, 3.0 µg each of H91A Hin47 + rHMW and increasing amounts of rHia;

Figure 2 contain bar graphs A and B, illustrating the anti-HMW immune responses for H91A Hin47 + rHMW + rHia combination vaccines in mice. Panel A, 0.3 µg each of H91A Hin47 + rHMW and increasing amounts of rHia. 15 Panel B, 3.0 µg each of H91A Hin47 + rHMW and increasing amounts of rHia. Asterisks indicate statistical significance;

Figure 3 contains bar graphs A, B, C and D illustrating the anti-Hia immune responses for H91A Hin47 + rHMW + rHia combination vaccines in mice. Panel A, 0.3 µg of rHia and increasing amounts of H91A Hin47 + 20 rHMW. Panel B, 1.0 µg of rHia and increasing amounts of H91A Hin47 + rHMW. Panel C, 3.0 µg of rHia and increasing amounts of H91A Hin47 + rHMW. Panel D, 10 µg of rHia and increasing amounts of H91A Hin47 + rHMW. Asterisks indicate statistical significance;

Figure 4 contains bar graphs A and B, illustrating the anti-H91A Hin47 immune responses for H91A Hin47 + rHMW + rHia combination vaccines in 25 guinea pigs- Panel A, 25 µg each of H91A Hin47 + rHMW and increasing amounts of rHia. Panel B, 50 µg each of H91A Hin47 + rHMW and increasing amounts of rHia;

Figure 5 contains bar graphs A and B, illustrating the anti-HMW immune 30 responses for H91A Hin47 + rHMW + rHia combination vaccines in guinea pigs. Panel A, 25 µg each of H91A Hin47 + rHMW and increasing amounts of rHia. Panel B, 50 µg each of H91A Hin47 + rHMW and increasing amounts of rHia;

Figure 6 contains bar graphs A, B and C, illustrating the anti-Hia immune responses for H91A Hin47 + rHMW + rHia combination vaccines in guinea pigs. Panel A, 25 µg of rHia and increasing amounts of H91A Hin47 + rHMW. Panel B, 50 µg of rHia and increasing amounts of H91A Hin47 + rHMW. Panel C, 100 µg of rHia and increasing amounts of H91A Hin47 + rHMW. Asterisks indicate statistical significance;

Figure 7 illustrates the protection of H91A Hin47 + rHMW + rHia combination vaccine in the chinchilla model of nasopharyngeal colonization compared to a mono-component or HMW vaccine and a two-component rHMW + H91A Hin47 and convalescent controls;

Figure 8 is a schematic illustration of a construction scheme for producing plasmid DS-2150-1 containing the gene encoding the H91 Hin47 analog;

Figure 9 is a schematic illustration of a construction scheme for producing plasmid BK-76-1-1 containing the hmw1ABC gene cluster from NTHi strain 12;

Figure 10 is a schematic illustration of a construction scheme for producing plasmid BK-96-2-11 containing the gene encoding N-truncated V38 Hia from NTHi strain 11;

Figure 11 shows the oligonucleotides used to PCR amplify the strain 11 *hia* gene. Sense (5038.SL), SEQ ID NO: 2, encoded amino acids SEQ ID NO: 3; Antisense (5039.SL), SEQ ID NO: 4, complement SEQ ID NO: 5, encoded amino acids SEQ ID NO: 6;

Figure 12 shows the oligonucleotides used to PCR amplify the 5'-fragments to produce the truncated gene. V38 truncation: Sense (5526.SL): SEQ ID NO: 7, encoded amino acids SEQ ID NO: 8; Anti-sense (5528.SL) SEQ ID NO: 9; complement SEQ ID NO: 10, encoded amino acids SEQ ID NO: 11; and

Figure 13 shows the protection afforded by the H91A Hin47 + rHMW rHia combination vaccine in the chinchilla model of intrabulla challenge. The protection afforded by the three-component vaccine is compared to that for a mono-component H91A Hin47 vaccine, a two-component rHMW and H91A Hin47 vaccine are convalescent controls.

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518 Rec'd PCT/PTO 31 AUG 2001  
09/914622GENERAL DESCRIPTION OF THE INVENTION

The production and purification of recombinant *H. influenzae* antigens rHMW, rHia and H91A Hin47 have been fully described in the aforementioned US Patent Applications Nos. 09/167,568 (WO 00/20609), 09/268,347 (WO 00/55191) and the aforementioned US Patent No. 5,506,159, respectively.

Colonization of the nasopharynx is the first step in disease development for many bacterial or viral pathogens and vaccines containing adhesin molecules should protect against this first step in disease progression. The high molecular weight (HMW) proteins, found in approximately 75% of non-typeable *H. influenzae*, have been shown to be adhesins that are protective against colonization when administered in a vaccine composition. The HMW proteins are not present in encapsulated *H. influenzae* strains or in about 25% of non-typeable *H. influenzae* strains, thus they are not sufficient for a fully-effective vaccine having strain-wide protectivity.

The Hia/Hsf proteins also have been shown to be adhesins and are present in all encapsulated *H. influenzae* strains and in most of those non-typeable *H. influenzae* strains which do not produce HMW proteins. The rHia protein is protective against colonization by NTHi and against bacteremia caused by *H. influenzae* type a and type b organisms. There is a small percentage of NTHi strains that produce neither HMW nor Hia proteins.

The HtrA protein or Hin47 is found in all encapsulated and non-typeable *H. influenzae* strains. Hin47, or its non-proteolytic H91A Hin47 mutant, is protective against bacteremia caused by *H. influenzae* type b and otitis media caused by non-typeable *H. influenzae*, but it does not prevent colonization. A combination vaccine comprising rHMW, rHia and H91A Hin47 antigens may be formulated to protect against *H. influenzae* disease, including otitis media. Such combination is provided herein.

The composition of multi-component vaccines is critical for maximum efficiency. The vaccine components must be compatible and they must be combined in appropriate ratios to avoid antigenic interference and optimize any possible synergies. If administered with other established vaccines, they must not interfere with the protection afforded by the vaccine against other disease(s).

The preparation, immunogenic and protective properties of a two-component rHMW + H91A Hin47 vaccine have been described in US Patent Application No. 09/210,995 filed December 15, 1998 (WO 00/35477), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference.

Various antigen ratios were compared for the three component H91A Hin47 + rHMW + rHia vaccine, in two animal species. There was no affect on the anti-H91A Hin47 response with increasing amounts of rHia. Antigenic interference was observed in mice for the anti-rHMW response, when a 0.3 µg dose of each of H91A Hin47 + rHMW was mixed with increasing doses of rHia. However, at a 3.0 µg dose of each of H91A Hin47 + rHMW, there was no suppression of the anti-rHMW response with increasing amounts of rHia. Although there was a transient suppression of the anti-Hia response on day 42 when a 0.3 µg dose was combined with 3 µg each of H91A + rHMW, this effect was not significant by day 56. In guinea pigs, the anti-H91A Hin47 and anti-rHMW responses were not effected by the addition of rHia. However, there appeared to be a small, but statistical, effect on the anti-Hia response in the presence of H91A Hin47 + rHMW for the booster immunizations. These data indicate that the composition of the three component vaccine is critical to achieve a good immune response to all components.

Referring to Fig. 1, there is illustrated the antibody response in mice to the H91A Hin47 antigen of a three-component H91A Hin47 + rHMW + rHia vaccine. High antibody titers were achieved with all vaccine combinations at the final bleed. Referring to Fig. 2, there is illustrated the antibody response in mice to the rHMW antigen of a three-component H91A Hin47 + rHMW + rHia vaccine. At the 3.0 µg dose of each of H91A Hin47 + rHMW, there are high titers of anti-rHMW antibodies found in the final bleed sera irrespective of the amount of rHia in the vaccine composition. However, at the 0.3 µg dose of each of H91A Hin47 + rHMW, the anti-rHMW titers are dramatically reduced with increasing amounts of rHia added. Referring to Fig. 3, at the 0.3 µg dose of rHia, there is a suppressive effect on the anti-rHia immune response on day 42 with increasing amounts of H91A Hin47 + rHMW. However, this effect is lost by day 56 and is not observed with higher doses, where there is no consequence on the immune response.

Referring to Fig. 4, there is illustrated the antibody response in guinea pigs to the H91A Hin47 antigen of a three component H91A Hin47 + rHMW + rHia vaccine. High antibody titers were achieved with all vaccine combinations at the final bleed and no synergistic or suppressive effects were observed. Referring to 5 Fig. 5, there is illustrated the antibody response in guinea pigs to the rHMW antigen of a three component H91A Hin47 + rHMW + rHia vaccine. High antibody titers were achieved with all vaccine combinations at the final bleed and no synergistic or suppressive effects were observed. Referring to Fig. 6, there is 10 illustrated the antibody response in guinea pigs to the rHia antigen of a three component H91A Hin47 + rHMW + rHia vaccine. High antibody titers were achieved with all vaccine combinations at the final bleed.

Referring to Fig. 7, there is illustrated the protection afforded by a three-component H91A Hin47 + rHMW + rHia vaccine in the nasopharyngeal colonization model compared to the protection afforded by the rHMW component alone or a two component H91A Hin47 + rHMW vaccine. The three component vaccine is highly protective, indicating that the addition of more antigens has not effected the protective ability of the rHMW component. 15

Referring to Figure 13, there is illustrated the protection afforded by the three-component H91A Hin47 + rHMW + rHia vaccine against middle ear infection in a chinchilla model. The protection is comparable to that afforded by a mono-component H91A Hin47 vaccine and a two-component H91A Hin47 + rHMW vaccine. 20

#### BIOLOGICAL DEPOSITS

Certain vectors that contain nucleic acid coding for a high molecular weight protein of a non-typeable strain of *Haemophilus* that are described and referred to herein have been deposited with the America Type Culture Collection (ATCC) located at 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, pursuant the Budapest Treaty and prior to the filing of this application. Samples of the deposited vectors will become available to the public and all 25 restrictions imposed or access to the deposits will be received upon grant of a patent based on this United States patent application. In addition, the deposit will 30 be replaced if viable samples cannot be dispensed by the Depository. The

invention described and claimed herein is not limited in scope by the biological materials deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar vectors that contain nucleic acid which encodes equivalent or similar antigens as described in this application  
5 are within the scope of the invention.

Deposit Summary

Plasmid	ATCC	Deposit Date
BK-76-1-1	203261	September 25, 1998
BK-96-2-11	203771	February 11, 1999

10

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and  
15 substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, immunology and fermentation technology used, but not explicitly described in this disclosure and  
20 these Examples, are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example describes the preparation of a H91A Hin47 vaccine component.

A H91A Hin47 mutant was prepared as described in the aforementioned US Patent No. 5,506,139 and as shown schematically in Figure 8. Briefly, an oligonucleotide 5'ATCAATAACAGCATTATTGGT3' (SEQ ID NO: 1) was synthesized which can change the Histidine residue at position 91 to an Alanine (ref. 19).

Plasmid JB-1276-1-2 is a pUC-based plasmid containing the T7/*hin47* gene on an *EcoR* I fragment and was used to clone the *hin47* gene into M13mp18 for site-directed mutagenesis with the *In Vitro* Site-Directed Mutagenesis kit from Amersham. The preparation of plasmid JB-1276-1-2 is described in USP 5,506,139. The mutation of the His91 codon to Ala91 was confirmed by local sequencing. The H91A mutant *hin47* gene was subcloned into pT7-7 to generate plasmid DS-1277-19 (Fig. 8).

The H91A Hin47 expression plasmid (DS-1277-19) utilizes ampicillin selection. The T7/H91A *hin47* gene was cloned into pBR328 so that tetracycline selection could be used. Vector DS-1312-12 was thus a pBR328-based plasmid which contained the T7/H91A *hin47* gene sequences between *EcoR* I and *Cla* I sites, having functional ampicillin and tetracycline resistance genes and containing a repeat of the *Hind* III - *BamH* I sequences which are found in both pBR328 and pEVvrfl.

A new plasmid based upon DS-1312-12 was constructed which utilizes kanamycin selection. The construction scheme is shown in Figure 8. Plasmid DNA from DS-1312-12 was digested with *Hind* III generating two fragments. The larger 5.9 kb fragment contained a promoterless *tetR* gene, the *ampR* gene and the T7/H91A *hin47* gene and was re-ligated on itself creating vector DS-2140-3. Plasmid DS-2140-3 was digested with *Pst* I and the *kanR* gene from plasmid pUC-4K (P-L Biochemicals) was inserted into the *Pst* I site, generating plasmid DS-2150-1 which is *kanR* and sensitive to both ampicillin and tetracycline.

Plasmid DNA from DS-2150-1 was prepared from a 50 mL culture using a protocol based upon the Holmes and Quigley procedure (ref. 21) and including extractions with phenol and chloroform. *E. coli* BL21(DE3) cells were made electrocompetent as follows. Briefly, 10 mL of overnight culture were inoculated into 500 mL of YT medium and the cells were grown at 37°C with shaking until they reached an  $A_{620}=0.540$ . The culture was chilled on ice for 30 min., spun at 5K rpm for 15 min., and the cell pellet resuspended in 500 mL ice cold sterile water. The cell suspension was centrifuged as before and the cell pellet resuspended in 250 mL ice cold sterile water. The cell suspension was spun again, and the cells were resuspended in 10 mL of 10% glycerol. The glycerol suspension was spun,

and the cells were resuspended in 1.5 mL of 10% glycerol, aliquotted as 40  $\mu$ L samples, and stored at -70°C.

One aliquot of electrocompetent BL21(DE3) cells was thawed on ice and approximately 9 ng of DS-2150-1 DNA was added. Samples were incubated on

5 ice for 3 min. then transferred to a -20°C BioRad\* Gene Pulser electrode cuvette and subjected to an electric pulse. 900  $\mu$ L of SOC medium were added and the mixture transferred to a culture tube where it was incubated at 37°C for 1 hour before being plated onto YT agar containing 25  $\mu$ g/mL kanamycin. The plate was incubated overnight at 37°C and single colonies were used for expression studies.

10 Individual clones were grown in NZCYM medium to an  $A_{600\text{ nm}}$  of approximately 0.3 and lactose was added to 1% to induce expression. Cells were grown for 4 hours, then harvested and analysed by SDS PAGE. Clone DS-2171-1-1 was chosen as a representative clone which expressed high levels of H91A Hin47.

15 The *E. coli* containing DS-2171-1-1 was grown in 2 X 2 L flasks containing 250 mL of ECGM (containing 8 g/L glucose, pH 6.5) and incubated by shaking at 37°C for approximately 9 hours in the dark at 250 rpm. The culture fluid (2 x 250 mL) was inoculated into a 10 L fermentor and the culture grown at 37°C. After approximately 10 hours of incubation, 1% lactose (final concentration) is added for induction, followed by an additional 4 hours incubation.

20 The culture fluid was harvested into sterile transfer bottles and concentrated and diafiltrated by cross-flow filtration against 50 mM Tris/HCl buffer, pH 8.0. The cells in the concentrate are lysed using a high-pressure homogenizer (2 passes at 15,000 psi) to release the H91A Hin47 protein. The cell debris was removed by centrifugation at 15,000 rpm for 1.5 hours. The supernatant was further clarified by centrifugation and filtered through a 0.22  $\mu$ m dead-end filter. Products may be stored frozen at -70°C until further processing.

25 Sodium chloride (NaCl) was added to the clarified sample to a final concentration of 100 mM. The sample was then purified on an anion exchange chromatography column (TMAE-Fractogel) equilibrated with 50 mM Tris pH 8.0

\* . Trademark

containing 100 mM NaCl. The H91A Hin47 protein was obtained in the run-through.

The aqueous layer was loaded onto a ceramic hydroxyapatite type 1 (CHTP-1) column equilibrated with 10 mM sodium phosphate buffer pH 8.0. The 5 column was then washed with 150 mM sodium phosphate buffer pH 8.0 and H91A Hin47 was eluted with 175 mM sodium phosphate buffer, pH 8.0 containing 1 M NaCl.

The H91A Hin47 purified protein was concentrated using a 10 kDa molecular weight cut-off membrane followed by diafiltration with approximately 10 volumes of phosphate buffered saline (PBS), pH 7.5.

The H91A Hin47 purified protein in PBS was passed through a Q600 Sartobind membrane adsorber. After passing the solution, the membrane was regenerated using 1.0 M KCl/1.0 M NaOH followed by washing with 1 M KCl then equilibrating with PBS. The process was repeated twice. The concentrated 15 diafiltered H91A Hin47 protein was sterile filtered through a 0.22 µm membrane filter. Sterile H91A Hin47 protein was adjuvanted with aluminum phosphate. The adsorbed purified concentrate was diluted to produce the adsorbed bulk at 100 µg/mL.

The concentration of the H91A Hin47 vaccine component was adjusted to 20 400 µg ml<sup>-1</sup> in PBS (pH 7.3) and was adjuvanted with aluminum phosphate to a final concentration of 3 mg ml<sup>-1</sup>. Different doses were prepared by diluting the stock with 3 mg ml<sup>-1</sup> of aluminum phosphate in PBS.

#### Example 2

This Example describes the preparation of a rHMW vaccine component.

The production and purification of the rHMW protein has been described 25 in the aforementioned copending United States Patent Application No. 09/167,568 filed October 7, 1998 (WO 00/20609) and is shown schematically in Figure 9.

Briefly, plasmid pHMW1-15 (ref. 13) contains a *Xba* I site within the T7 promoter sequence and a unique *BamH* I site within the coding sequence of the 30 mature HMW1A protein of non-typeable *Haemophilus* strain 12. The 1.8 kb *Xba* I-*BamH* I fragment of pHMW1-15 was deleted and replaced by an approximately 114 bp *Xba* I-*BamH* I fragment generated from oligonucleotides. The resultant 11.3 kb

plasmid, DS-1046-1-1, thus contains the T7 promoter joined in frame with the *hmw1ABC* operon that encodes the mature 125 kDa HMW1A protein (Fig. 9).

Plasmid DS-1046-1-1 contains the T7 *hmw1ABC* gene cassette and has a unique *Bgl* II site outside the coding region of the mature HMW1A gene. Plasmid

5 DS-2224-1-4 contains the *E. coli cer* gene located on a *BamH* I fragment. This fragment was isolated and ligated into the *Bgl* II site of plasmid DS-1046-1-1 to produce plasmid BK-35-4 (Fig. 9). The kanamycin resistance cassette was excised from pUC 4K by *Sal* I restriction and ligated into the *Sal* I restricted BK-35-4 plasmid to produce plasmid BK-76-1-1.

10 Plasmids were introduced into *E. coli* BL21(DE3) cells by electroporation using a BioRad apparatus. Strains were grown at 37°C in NZCYM medium to an optical density of  $A_{578}=0.3$ , then induced by the addition of lactose to 1.0% for 4 hours. Samples were adjusted to 0.2 OD/ $\mu$ l with SDS-PAGE lysis + loading buffer and the same amount of protein sample was loaded onto SDS-PAGE gels. Clone 15 BK-116-1-1 was selected as a representative clone for preparation of seed stocks.

Recombinant HMW protein was expressed as inclusion bodies in *E. coli*, and were purified by the same procedure (Figure 12). *E. coli* cell pellets from 500 ml culture were resuspended in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, and disrupted by sonication. The extract was centrifuged at 20,000 g for 20 30 min and the resultant supernatant was discarded. The pellet was further extracted, in 50 ml of 50 mM Tris-HCl, pH 8.0 containing 0.5% Triton\* X-100 and 10 mM EDTA, then centrifuged at 20,000 g for 30 min, and the supernatant was discarded. The pellet was further extracted in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 1 % octylglucoside, then centrifuged at 20,000 g for 30 min, and 25 the supernatant was discarded.

The resultant pellet, obtained after the above extractions, contains the inclusion bodies. The pellet was solubilized in 6 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. Twelve ml of 50 mM Tris-HCl, pH 8.0 was added to this solution and the mixture was centrifuged at 20,000 g for 30 30 min. The supernatant was precipitated with polyethylene glycol (PEG) 4000 at a final concentration of 7%. The resultant pellet was removed by centrifugation at 20,000 g for 30 min and the supernatant was precipitated by  $(NH_4)_2SO_4$  at 50%

\* - Trademark

saturation. After the addition of  $(\text{NH}_4)_2\text{SO}_4$ , the solution underwent phase separation with protein going to the upper phase, which was then subjected to centrifugation at 20,000 g for 30 min. The resultant pellet was dissolved in 2 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine HCl and 5 mM DTT and 5 the clear solution was purified on a Superdex 200 gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 2 M guanidine HCl. The fractions were analysed by SDS-PAGE and those containing the purified rHMW1 were pooled and dialysed overnight at 4°C against PBS, then centrifuged at 20,000 g for 30 min. The protein remained soluble under these conditions and 10 glycerol was added to the rHMW1 preparation at a final concentration of 20% for storage at -20°C.

The concentration of the rHMW vaccine component was adjusted to 400  $\mu\text{g ml}^{-1}$  in PBS (pH 7.3) and was adjuvanted with aluminum phosphate to a final concentration of 3  $\text{mg ml}^{-1}$ . Different doses were prepared by diluting the stock 15 with 3  $\text{mg ml}^{-1}$  aluminum phosphate in PBS.

Example 3

This Example illustrates the preparation of a rHia vaccine component.

The production and purification of the rHia protein has been described in the aforementioned copending United States Patent Application No. 09/268,347 20 filed March 16, 1999 (WO 00/55191) and is shown schematically in Figure 10.

Briefly, chromosomal DNA was purified from NTHi strain 11 and the full-length *hia* gene was PCR amplified using the oligonucleotides (5038.SL and 5039.SL) (Fig. 11). The PCR product contained an NdeI site at the 5' end and a BamHI site at the 3' end. This fragment was cloned into the NdeI/BamHI 25 restricted pT7-7 expression vector (ref.20)producing plasmid DS-2008-2-3 (Fig. 10).

PCR primers (5526.SL and 5528.SL) (Fig 12) were used to amplify a truncated *hia* gene fragment from the V38 site to the Sty I site of plasmid DS-2008-2-3, the resulting fragment was TA cloned into plasmid pCRII (Invitrogen) 30 to produce plasmid DS-2153-3-5. This plasmid was then restricted with Nde I and Sty I and this fragment was ligated to the Nde I/Sty I 5.7kb isolated vector fragment from DS-2008-2-3 to produce plasmid DS-2186-2-1.

Plasmid DS-2186-2-1 containing the V38 truncated *hia* gene, was restricted with Bgl II and BamH I to release the *rHia* gene. This fragment was isolated and cloned into the BglII restricted, CAP treated, plasmid BK-2-1-2, to produce plasmid BK 96-2-11. This plasmid now possesses a kanamycin resistance marker and the *E. coli cer* gene as well as the truncated V38 strain 11 *hia* gene.

The concentration of the *rHia* vaccine component was adjusted to 400 µg ml<sup>-1</sup> in PBS (pH 7.3) and was adjuvanted with aluminum phosphate to a final concentration of 3 mg ml<sup>-1</sup>. Different doses were prepared by diluting the stock 10 with 3 mg ml<sup>-1</sup> aluminum phosphate in PBS.

Example 4

This Example describes the combination of H91A Hin47 + rHMW + *rHia* as a three-component vaccine.

The preparation of a two-component vaccine comprising H91A Hin47 + 15 rHMW, has been described in the aforementioned copending United States Patent Application No. 09/210,995 filed December 15, 1998 (WO 00/35477). Briefly, vaccines were prepared that comprised combination of H91A Hin47 and rHMW by combining components on day 0, mixing overnight at 4°C and aliquotted on 20 day 1. The combined vaccines were stored at 4°C throughout the immunization period.

Vaccines were prepared that comprised the following combinations of *rHia* with the two component vaccine contained in Table II:

21

TABLE II

rHia→	0	0.3	1.0	3.0	10	25	50	100
2 COMPONENT ↓	·							
0		m	m	m	m	gp	gp	gp
0.3 + 0.3	m	m	m	m	m			
3.0 + 3.0	m	m	m	m	m			
25 + 25	gp					gp	gp	gp
50 + 50	gp					gp	gp	gp

Notes: 2 component refers to H91A Hin47 + rHMW

m indicates the vaccine was used to immunize mice.

gp indicates that the vaccine was used to immunize guinea pigs.

Vaccine components were combined on day 0, mixed overnight at 4°C, and aliquotted on day 1. The multi-component vaccines were stored at 4°C throughout the immunization period.

## 5 Example 5

This Example describes the analysis of the immunogenicity of the multi-component vaccines in animals.

The immunogenicity of a two-component vaccine comprising H91A Hin47 + rHMW, has been described in the aforementioned copending United States Patent Application No. 09/210,995 filed December 15, 1998 (WO 00/35477).

Groups of five BALB/c mice (Charles River, Quebec) were immunized subcutaneously (s.c.) on days 1, 29 and 43 with one of the mouse vaccines described in Example 4. Blood samples were taken on days 0, 14, 28, 42, and 56.

15 Groups of five Hartley outbred guinea pigs (Charles River, Quebec) were immunized intramuscularly (i.m.) on days 1, 29 and 43 with one of the guinea pig vaccines described in Example 4. Blood samples were taken on days 0, 14, 28, 42, and 56.

Anti-H91A Hin47, anti-rHMW, and anti-rHia IgG antibody titers were determined by antigen specific enzyme linked immunosorbent assays (ELISAs). Microtiter wells (Nunc-MAXISORB\*, Nunc, Denmark) were coated with 50 µl of protein solution ( $0.4 \mu\text{g ml}^{-1}$  for H91A Hin47,  $0.4 \mu\text{g ml}^{-1}$  for rHMW, or  $0.4 \mu\text{g ml}^{-1}$  for rHia).

\* - Trademark

ml<sup>-1</sup> for rHia). The secondary antibodies used were affinity-purified F(ab'), fragments of goat anti-mouse IgG (Fc-specific) or anti-guinea pig IgG (Fc-specific) antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs, Mississauga, Ontario). The reactions were developed 5 using tetramethylbenzidine (TMB/H202, ADI, Mississauga, Ontario) and absorbancies were measured at 450 nm (using 540 nm as a reference wavelength) in a Flow Multiskan MCC microplate reader (ICN Biomedicals, Mississauga, Ontario). The reactive titer of an antiserum was defined as the reciprocal of the dilution consistently showing a two-fold increase in adsorbance over that obtained 10 with the prebleed serum sample.

The results of the immunogenicity studies are illustrated in Figures 1 to 6. As shown in Figure 1, the final bleed sera obtained from mice immunized with 0.3 ug each of H91A Hin47 + rHMW or with 3.0 µg each of H91A Hin47 + rHMW and 0, 0.3, 1.0, 3.0 or 10 µg of rHia all had equivalent high antibody titers to 15 H91A Hin47. These data show that there is neither a synergistic nor an interfering effect on the anti-H91A Hin47 antibody titer with added rHia.

As shown in Figure 2, panel A, the final bled sera obtained from mice immunized with 0.3 µg each of H91A Hin47 + rHMW and 0, 0.3, 1.0, 3.0 or 10 µg of rHia had significantly reduced anti-HMW titers with increased amounts of 20 rHia added. These data indicate that at low concentrations of H91A Hin47 + rHMW, there is suppression of the anti-HMW antibody response caused by the addition of rHia. When mice are immunized with 3 µg each of H91A, Hin47 + rHMW, and 0, 0.3, 1.0, 3.0 or 10 µg of rHia; no affect was observed and high titers of anti-rHMW antibodies were obtained in the final bleed sera (Fig. 2, panel 25 B).

As shown in Figure 3, panel A, the addition of 3.0 µg each of H91A Hin47 + rHMW to 0.3 µg of rHia, had a transient suppressive effect on the anti-rHia response at day 42, that disappeared by day 56. However, as shown in Figure 3, panels B to D, there was no suppressive effect observed at higher doses of rHia.

30 As shown in Figure 4, panels A and B, the final bleed sera from guinea pigs immunized with 25 µg each of H91A Hin47 + rHMW or 50 µg each of H91A Hin47 + rHMW and 0, 25, 50 or 100 µg of rHia all had high titers of anti-H91A

Hin47 antibodies. These data indicate that there was neither a synergistic nor a suppressive affect on the anti-H91A Hin47 antibody response in the presence of the three antigens.

As shown in Figure 5, panels A and B, the final bleed sera from guinea pigs immunized with 25 µg each of H91A Hin47 rHMW or 50 µg each of H91A Hin47 + rHMW and 0, 25, 50 or 100 µg of rHia all had high titers of anti-rHMW antibodies. These data indicate that there was neither a synergistic nor a suppressive affect on the anti-rHMW antibody response in the presence of the three antigens.

10 As shown in Figure 6, panels A to C, the final bleed sera from guinea pigs immunized with 25, 50 or 100 µg of rHia with or without added H91A Hin47 + rHMW, all had high titer anti-rHia antibodies. There was, however, a slight but statistical inhibition of the anti-rHia response after booster doses containing all three antigens.

15 **Example 6**

This Example describes the protective ability of a multi-component vaccine in animal models of disease.

In young chinchillas, it has been demonstrated that nasopharyngeal colonization with non-typeable *H. influenzae* leads to otitis media (ref. 14). rHMW 20 is partially protective in a chinchilla nasopharyngeal colonization challenge model, as described in copending US Patent Application No. 09/167,568 (WO 00/20609). In this model, animals are immunized i.m. on days 0, 14 and 28 with 25, 50 or 100 µg of rHMW, adsorbed to alum, and challenged on day 44 with 10<sup>8</sup> cfu of live bacteria delivered intranasally (50 µl per nares).

25 Nasopharyngeal lavage is performed 4 days post challenge using 1 ml of sterile saline as wash. 25 µl of wash is plated onto chocolate agar in the presence of streptomycin and the plates incubated at 37°C for 24 h. (The challenge strain was made streptomycin resistant by serial passaging, in order to facilitate the quantitation of recovered bacteria in the presence of natural flora that are killed by 30 the streptomycin.) Convalescent animals or those mock-immunized with alum alone, are used as controls. For the multi-component vaccine study, 50 µg each of rHMW, rHia, and H91A Hin47 were mixed as described in Example 4 and

chinchillas were immunized as described above.

The results of the protection study are shown in Figure 7 which indicates that there is still excellent protection afforded in the nasopharyngeal colonization challenge model by the combination of rHMW + rHia + H91A Hin47.

5 In addition, as seen in Figure 13, the three-component vaccine afforded partial protection in the intrabulla challenge model. This model has been described previously (ref. 19). Young chinchillas are immunized i.m. on days 0, 14 and 28 with 50 µg of each of the antigens tested, adsorbed to alum. The chinchillas were then challenged on day 44 with 350 cfu of live organisms delivered into the  
10 middle ear space via the epitympanic bulla. Animals are then monitored by tympanometry and middle ear fluid is collected 4 days post-challenge, mixed with 200 µl of BHI medium and dilution plated onto chocolate agar plates that are incubated for 24 hours at 37°C. Convalescent animals or those immunized with alum alone, are used as controls. For the multi-component vaccine study, 50 µl  
15 each of H91A Hin47, rHMW and rHia were mixed as described in Example 4.

#### SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a multi-component vaccine against *Haemophilus influenzae* having a wide spectrum of efficacy and comprising three different antigens of *Haemophilus influenzae*, two  
20 of which different antigens are adhesin. Modifications are possible within the scope of this invention.

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**CLAIMS**

1. An immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae*, comprising:  
at least three different antigens of *Haemophilus influenzae*, at least two of which different antigens is an adhesin and the other of which is not an adhesin.
2. The immunogenic composition of claim 1 wherein one of said antigens which is an adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae*.
3. The immunogenic composition of claim 2 wherein said HMW protein is a HMW1 or HMW2 protein of the non-typeable strain of *Haemophilus influenzae*.
4. The immunogenic composition of claim 1 wherein one of the antigens which is an adhesin is a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae* or a *Haemophilus influenzae* surface fibril (Hsf) protein of a typeable strain of *Haemophilus influenzae*.
5. The immunogenic composition of claim 1 wherein the antigen of *Haemophilus influenzae* which is not an adhesin is a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae*.
6. The immunogenic composition of claim 5 wherein the non-proteolytic heat shock protein of a strain of *Haemophilus influenzae* is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of natural Hin47 protein.
7. The immunogenic composition of claim 1, wherein one of said antigens which is an adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae* and the other of the antigens which is an adhesin is a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae* or a *Haemophilus influenzae* surface fibril (Hsf) protein of a typeable strain of *Haemophilus influenzae*.
8. An immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae*, which comprises:
  - (a) an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of natural Hin47 protein,

(b) a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae*, and

(c) a high molecular weight (HMW) protein of a strain of non-typeable *Haemophilus influenzae*.

9. The composition of claim 8 wherein said Hin47, Hia and HMW proteins are present in amounts which do not impair the individual immunogenicities of the proteins.

10. The composition of claim 9 wherein said analog of Hin47 protein is one in which at least one amino acid of the natural Hin47 protein contributing to protease activity has been deleted or replaced by a different amino acid and which has substantially the same immunogenic properties as natural Hin47 protein.

11. The composition of claim 10 wherein said at least one amino acid is selected from the group consisting of amino acids 91, 121 and 195 to 201 of natural Hin47 protein.

12. The composition of claim 11 wherein Serine-197 is replaced by alanine.

13. The composition of claim 11 wherein Histidine-91 is replaced by alanine, lysine or arginine.

14. The composition of claim 13 wherein Histidine-91 is replaced alanine.

15. The composition of claim 11 wherein Asp-121 is replaced by alanine.

16. The composition of claim 9 wherein said Hia protein is produced recombinantly.

17. The composition of claim 16 wherein said recombinantly-produced Hia protein is an N-terminal truncation V38 rHia.

18. The composition of claim 9 wherein said HMW protein is an HMW1 or HMW2 protein of a non-typeable strain of *Haemophilus influenzae*.

19. The composition of claim 18 wherein the HMW1 and HMW2 proteins are produced recombinantly.

20. The composition of claim 18 wherein said HMW1 and HMW2 proteins are derived from the respective strain of non-typeable *Haemophilus influenzae* and possess respective molecular weights as set forth in the following Table:

<u>Molecular Weight(kDa)</u>	<u>Non-typeable <i>H. influenzae</i> Strain</u>					
	12	JoyC	K21	LCDC2	PMH1	15
Mature Protein:	HMW1	125	125.9	104.4	114.0	102.4
	HMW2	120	100.9		111.7	103.9

21. The composition of claim 8 further comprising an adjuvant.

22. The composition of claim 21 wherein said adjuvant is aluminum hydroxide or aluminum phosphate.

23. The composition of claim 8 comprising

- (a) about 25 to about 100 µg of the Hin47 protein analog, and
- (b) about 25 to about 100 µg of the Hia protein, and
- (c) about 25 to about 100 µg of the HMW protein.

24. The composition of claim 8 further comprising at least one additional antigenic component for conferring protection against infection caused by another pathogen.

25. The composition of claim 24 wherein said at least one additional antigenic component is selected from the group consisting of diphtheria toxoid, tetanus toxoid, pertussis antigens, non-virulent poliovirus and PRP-T.

26. The composition of claim 25 wherein said pertussis antigens are selected from the group consisting of pertussis toxoid, filamentous hemagglutinin, pertactin and agglutinogens.

27. A method of immunizing a host against disease caused by infection with *Haemophilus influenzae*, including otitis media, which comprises administering to the host an immunoeffective amount of a composition as claimed in claim 1 or 8.

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## Anti-H91AHin47 Antibody Titers in Mice

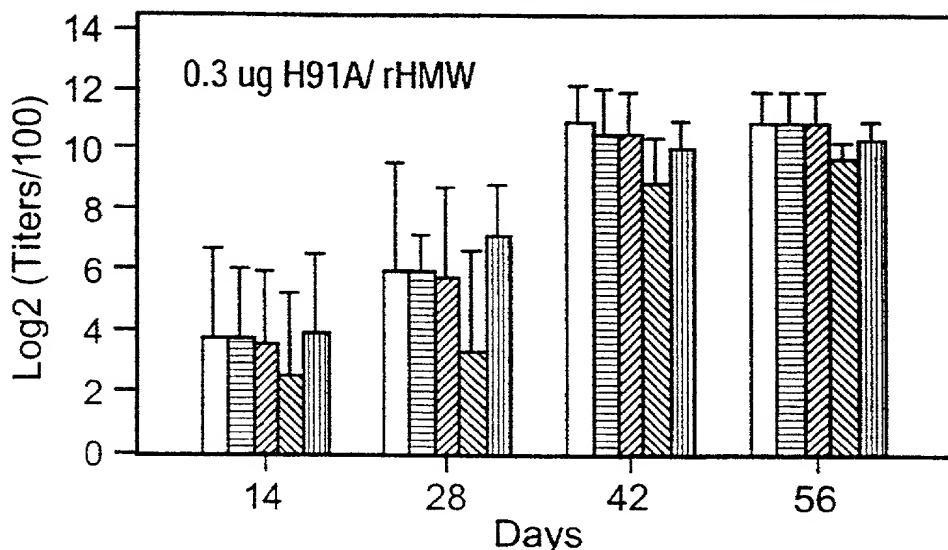


FIG.1A

## Anti-H91AHin47 Antibody Titers in Mice

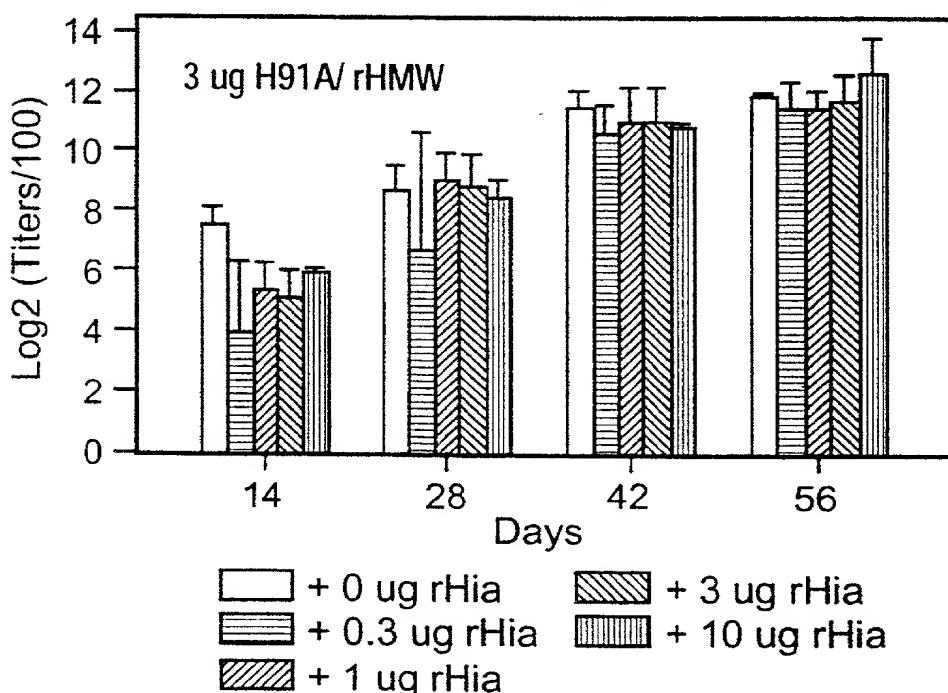


FIG.1B

Anti-rHMW Antibody Titers in Mice

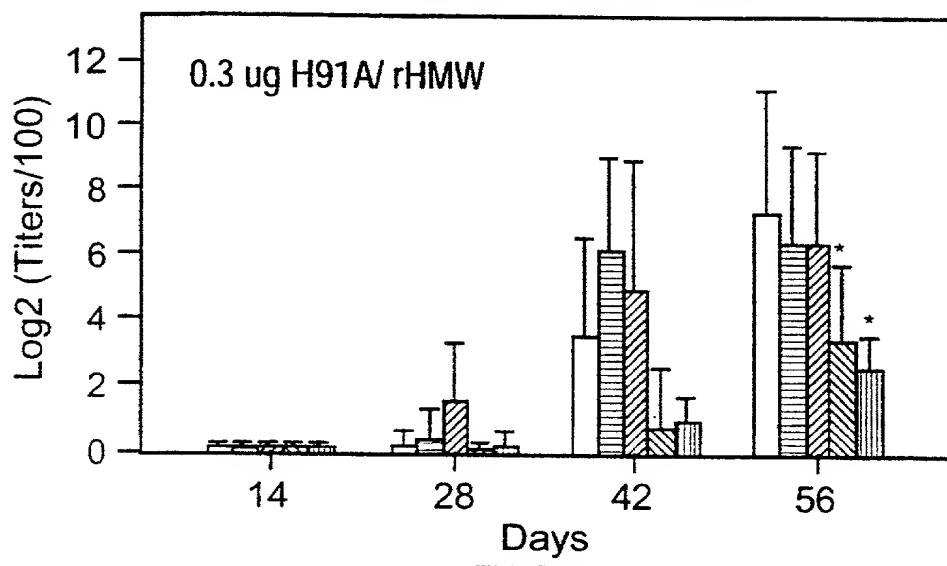


FIG.2A

Anti-rHMW Antibody Titers in Mice

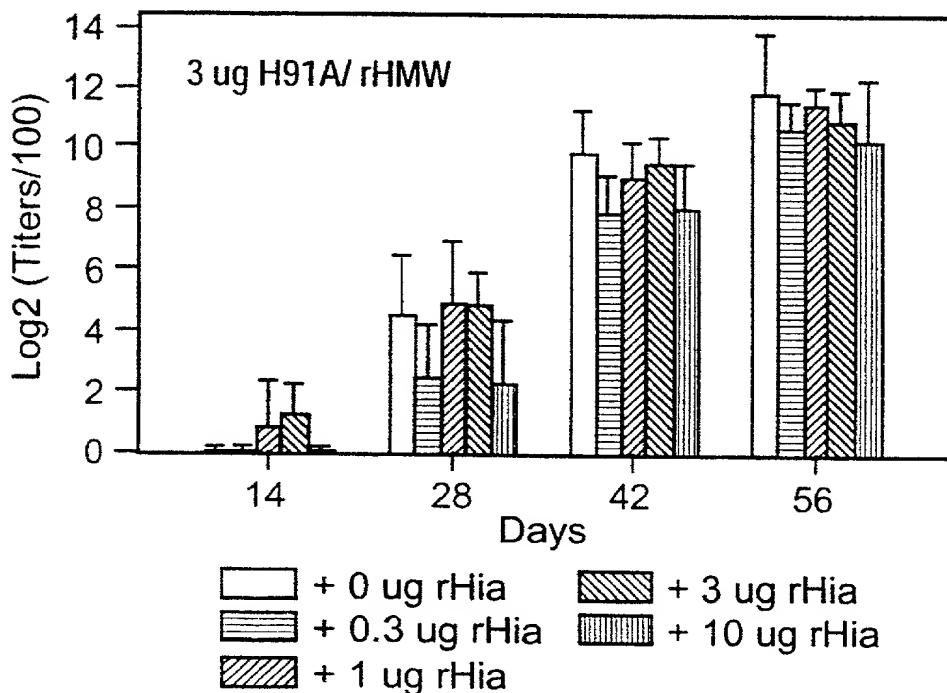


FIG.2B

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## Anti-rHia Antibody Titers in Mice

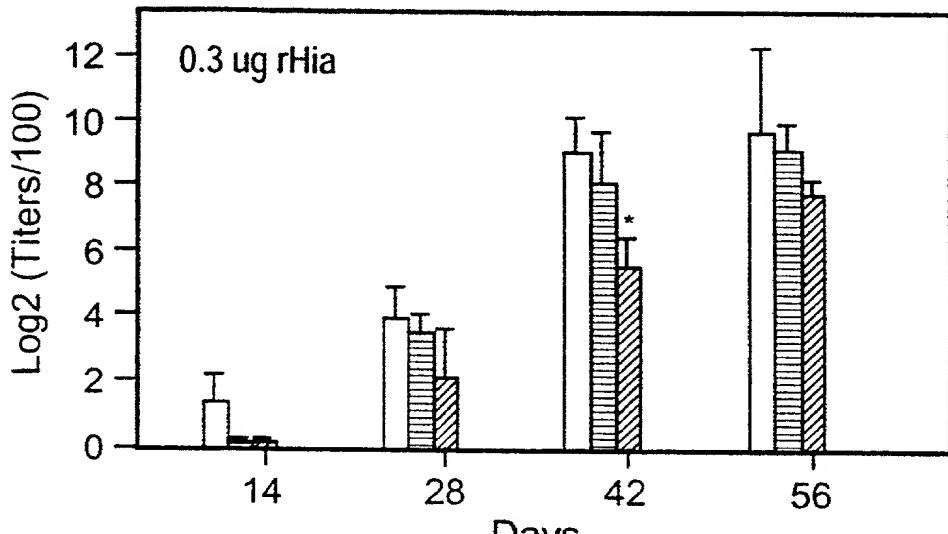


FIG.3A

## Anti-rHia Antibody Titers in Mice

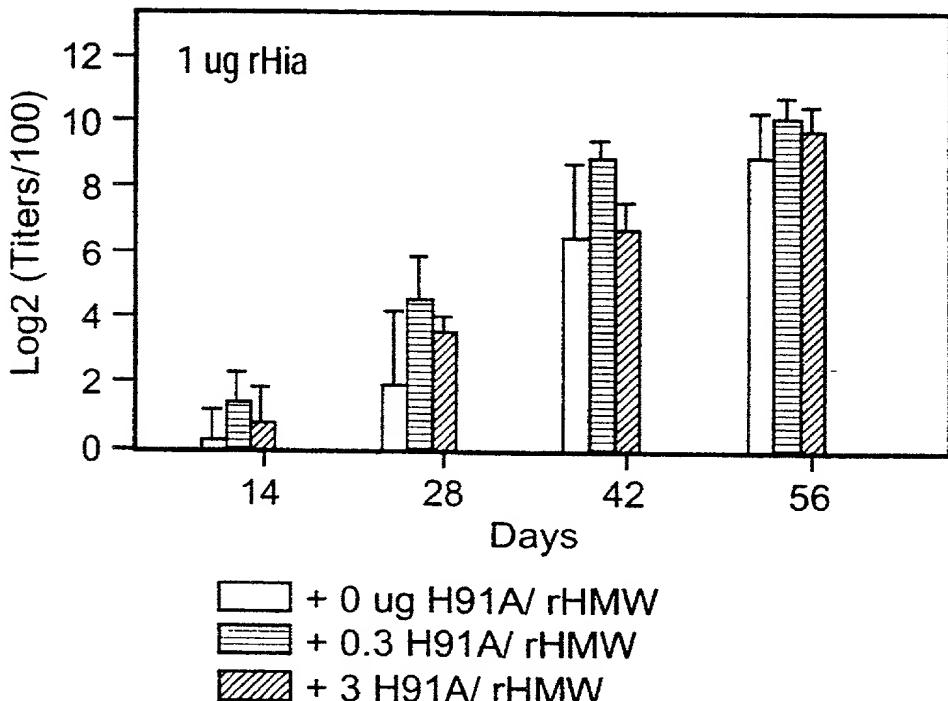


FIG.3B

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## Anti-rHia Antibody Titers in Mice

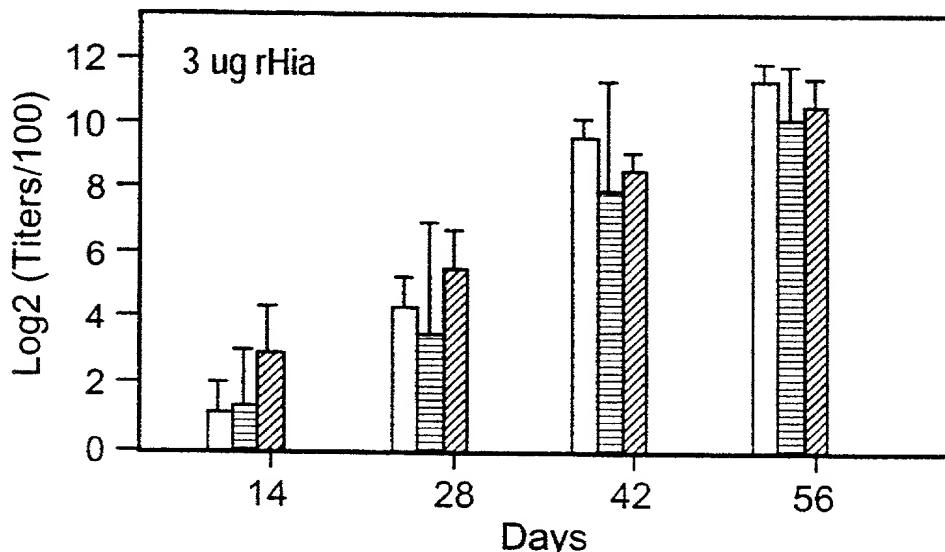
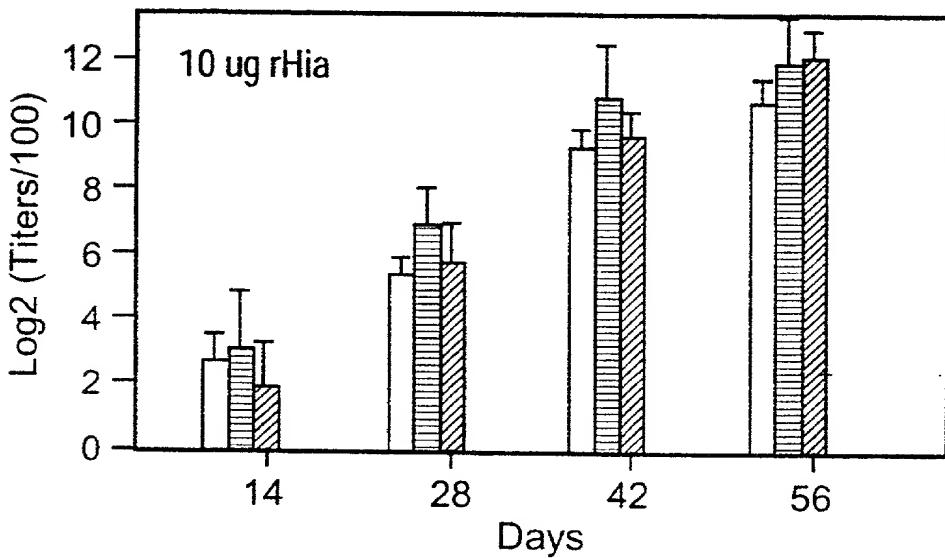


FIG.3C

## Anti-rHia Antibody Titers in Mice



- [White Box] + 0 ug H91A/ rHMW
- [Hatched Box] + 0.3 H91A/ rHMW
- [Dashed Box] + 3 H91A/ rHMW

FIG.3D

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## Anti-H91AHin47 Antibody Titers in Guinea Pigs

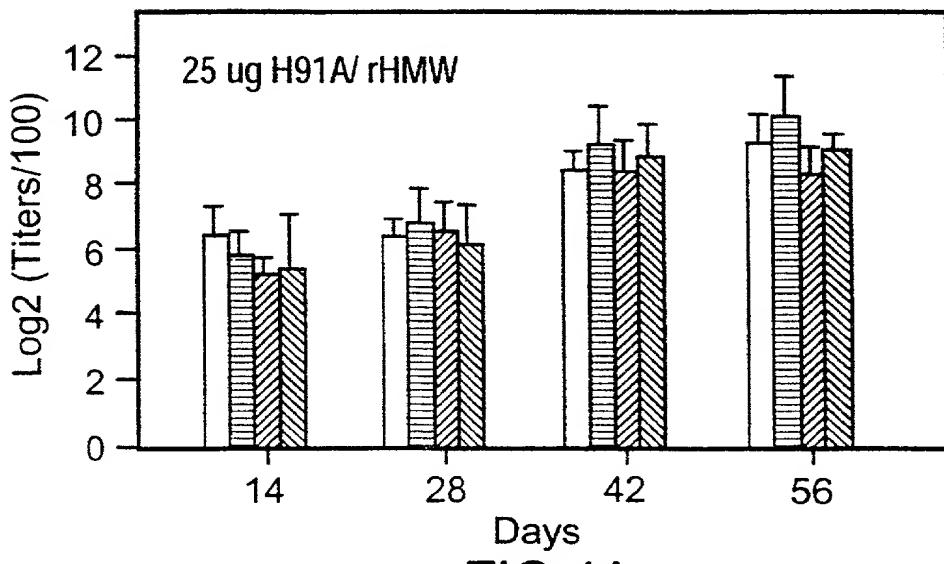


FIG.4A

## Anti-H91AHin47 Antibody Titers in Guinea Pigs

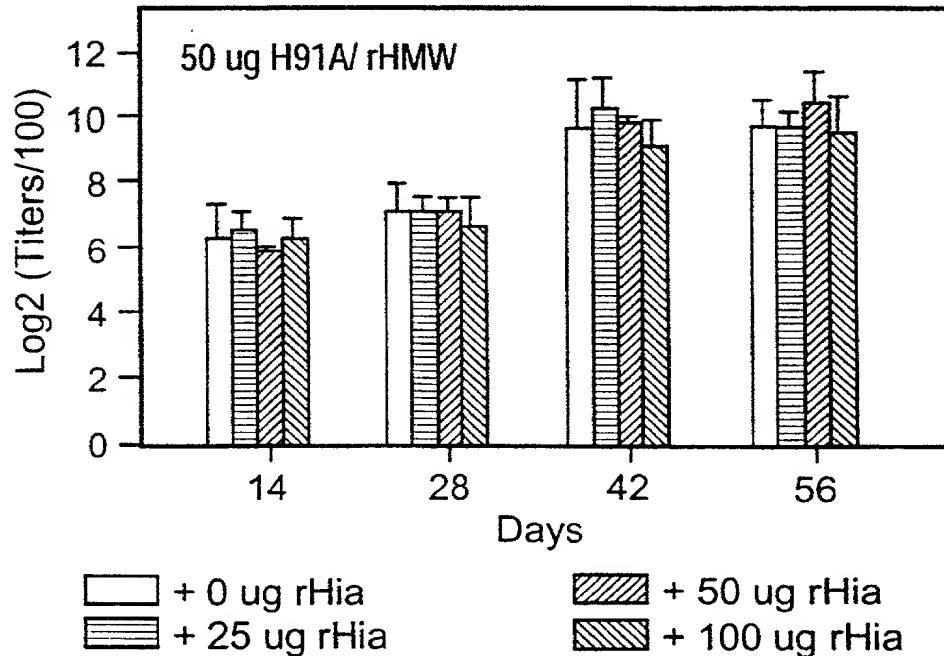


FIG.4B

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## Anti-rHMW Antibody Titers in Guinea Pigs

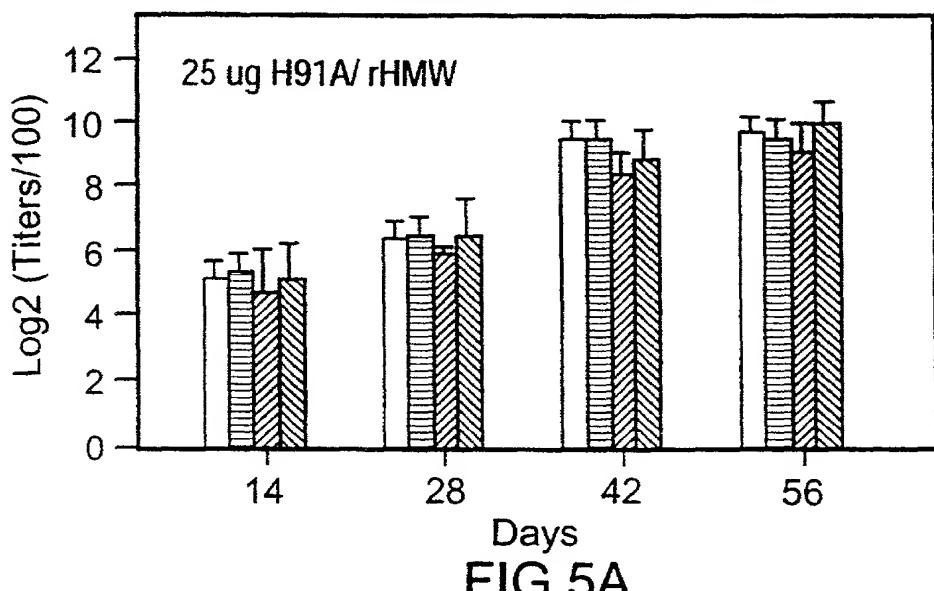


FIG.5A

## Anti-rHMW Antibody Titers in Guinea Pigs

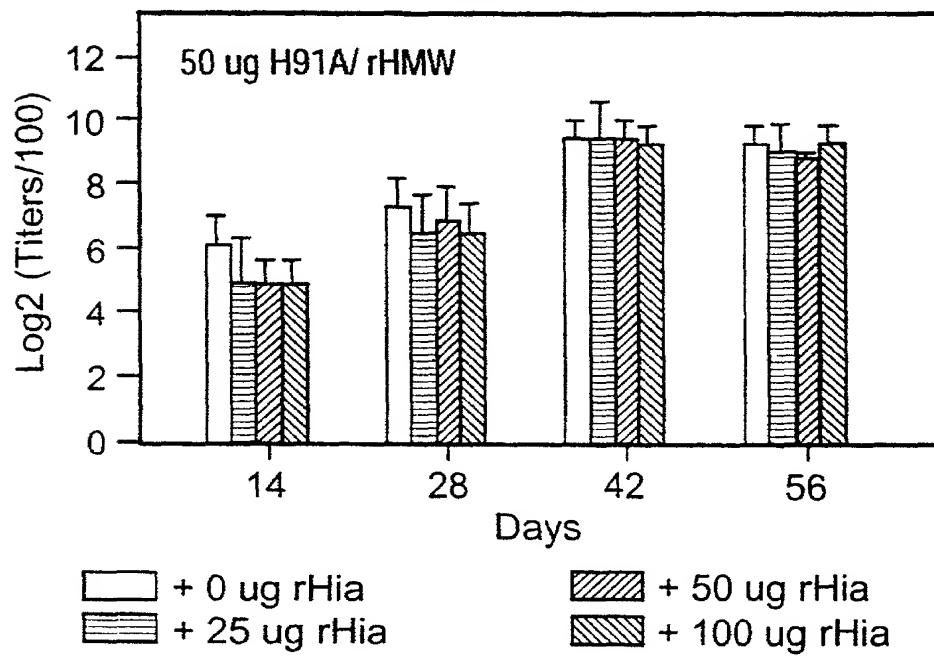
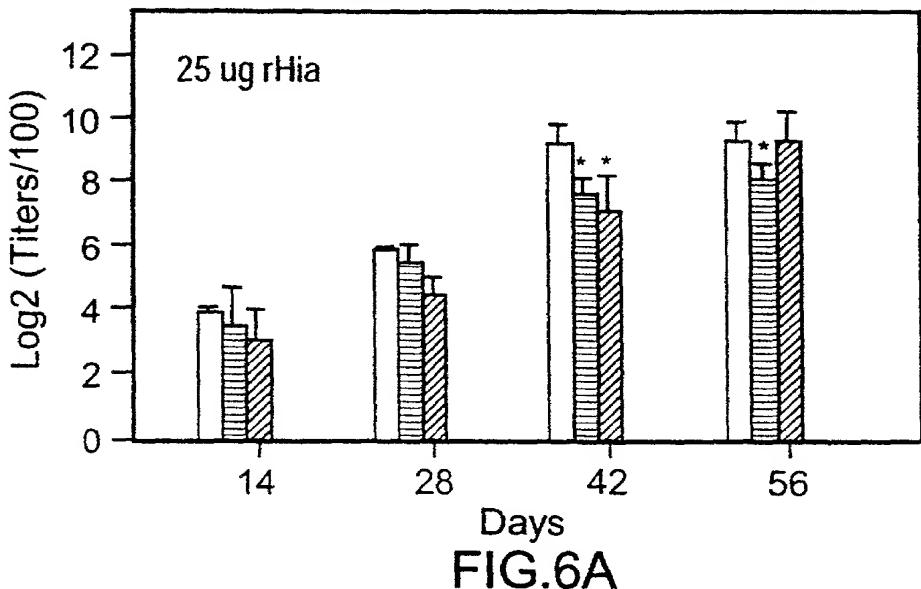


FIG.5B

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## Anti-rHia Antibody Titers in Guinea Pigs



## Anti-rHia Antibody Titers in Guinea Pigs

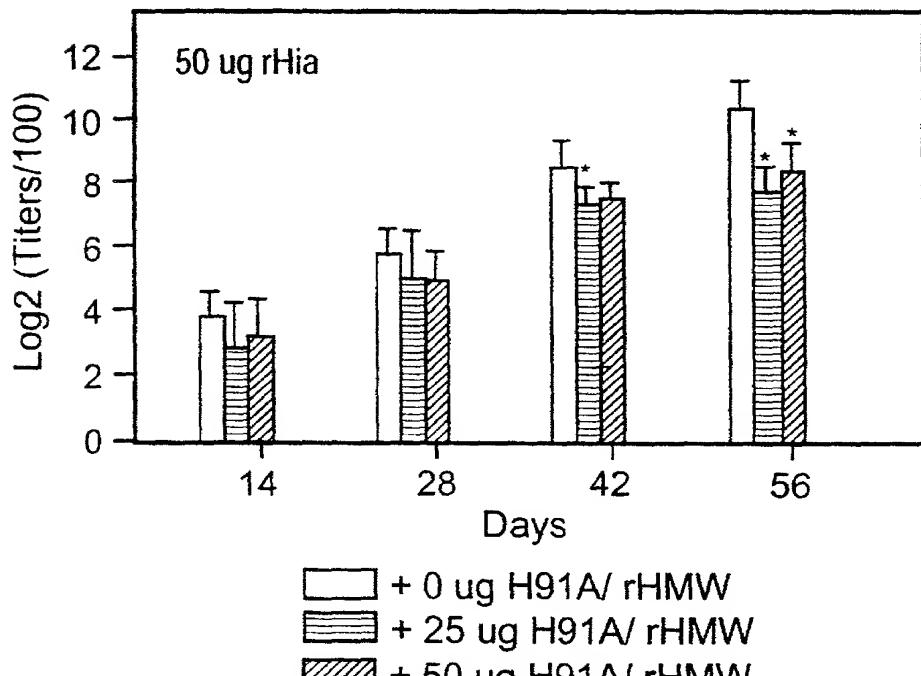


FIG.6B

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## Anti-rHia Antibody Titers in Guinea Pigs

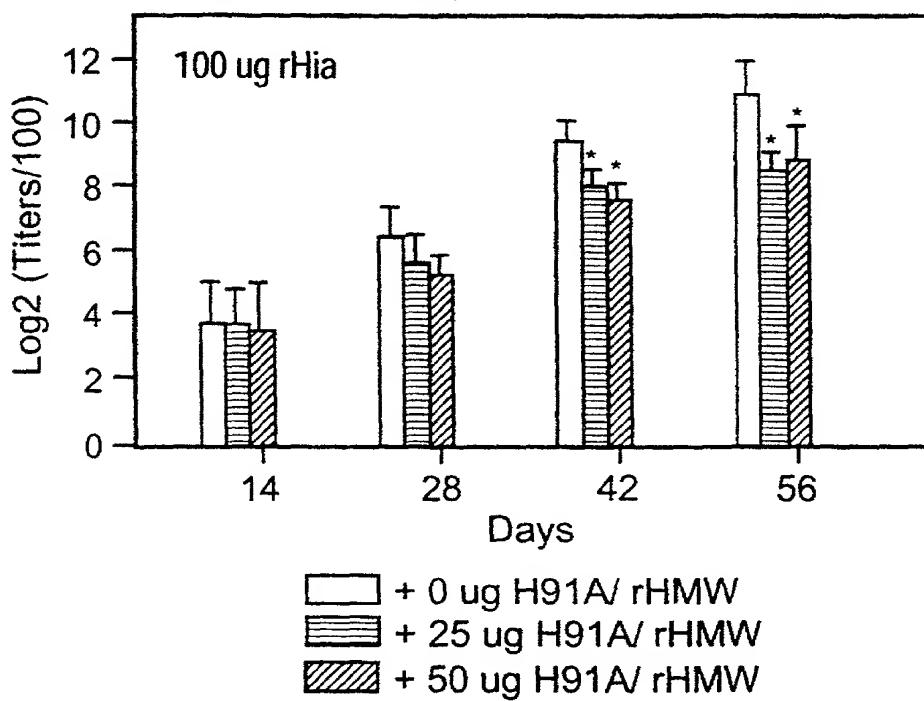


FIG.6C

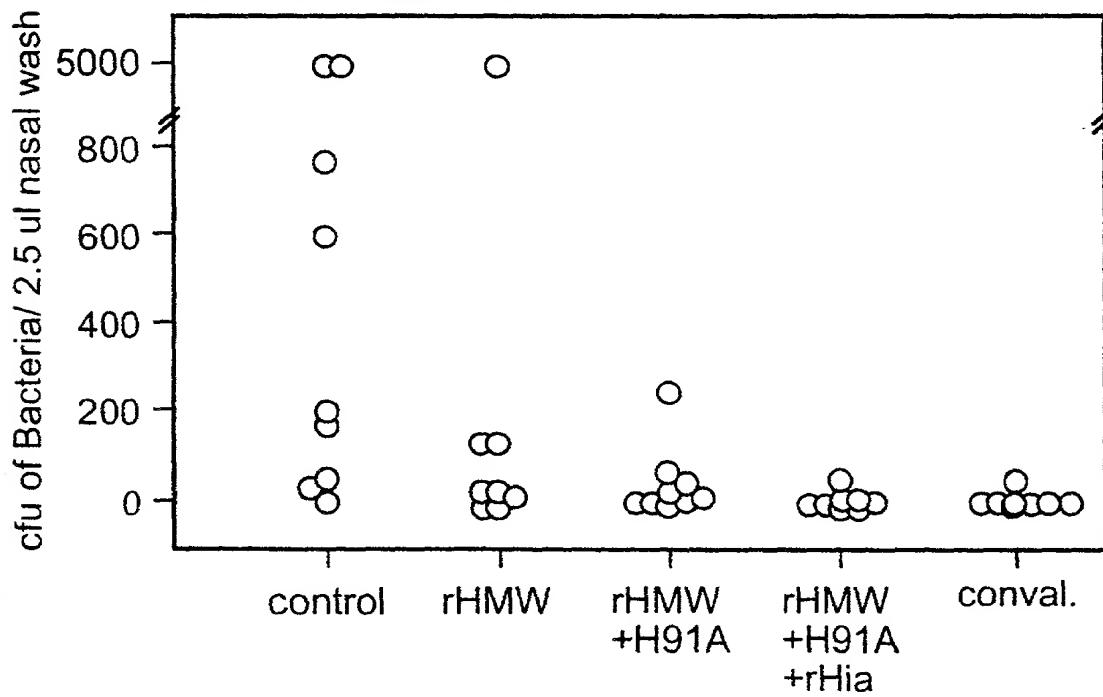
Protective Ability of A Three-component *H. influenzae* Vaccine Against NP Colonization with NTHi Strain 12 in Chinchillas

FIG.7

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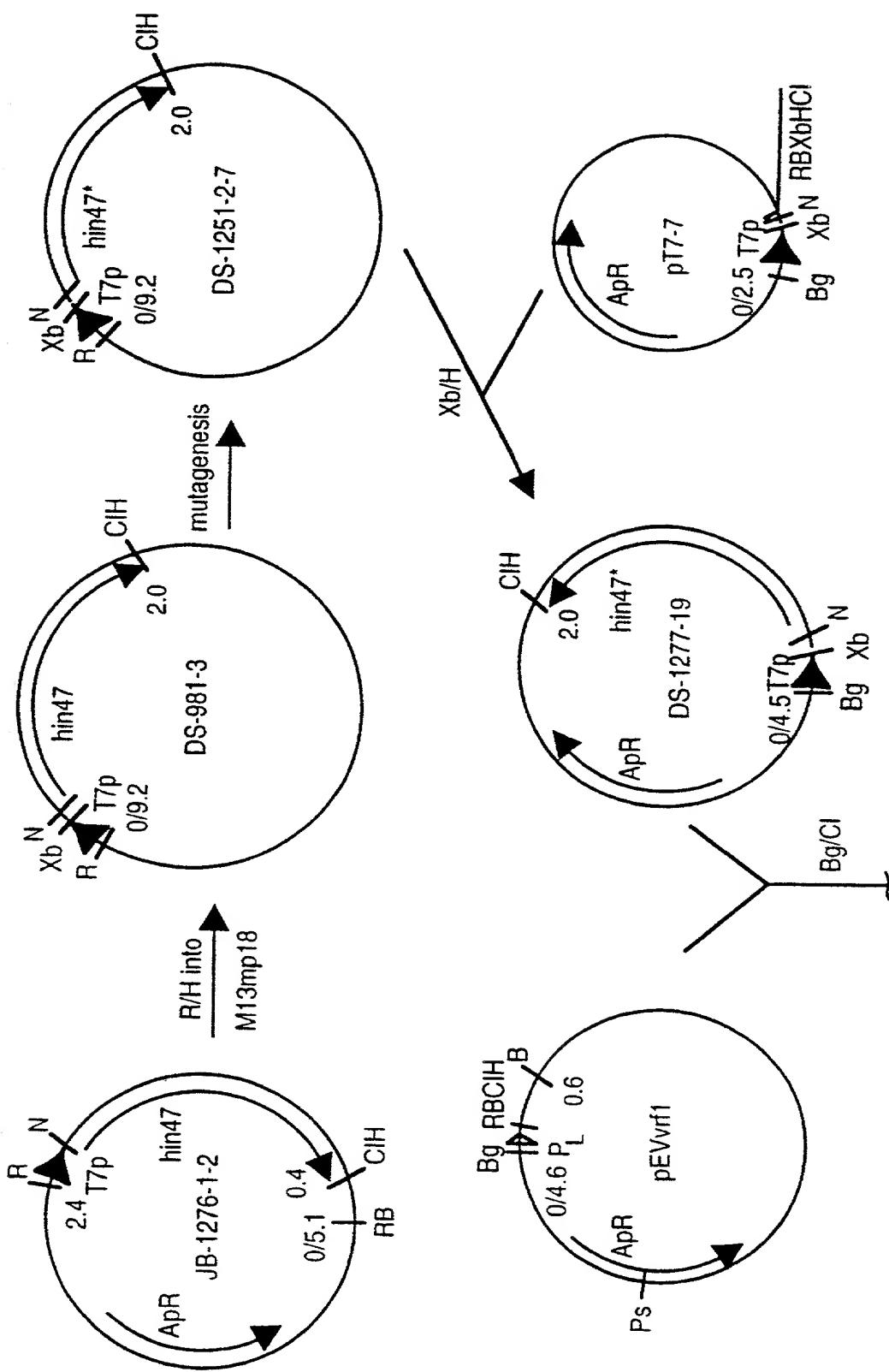


FIG.8A

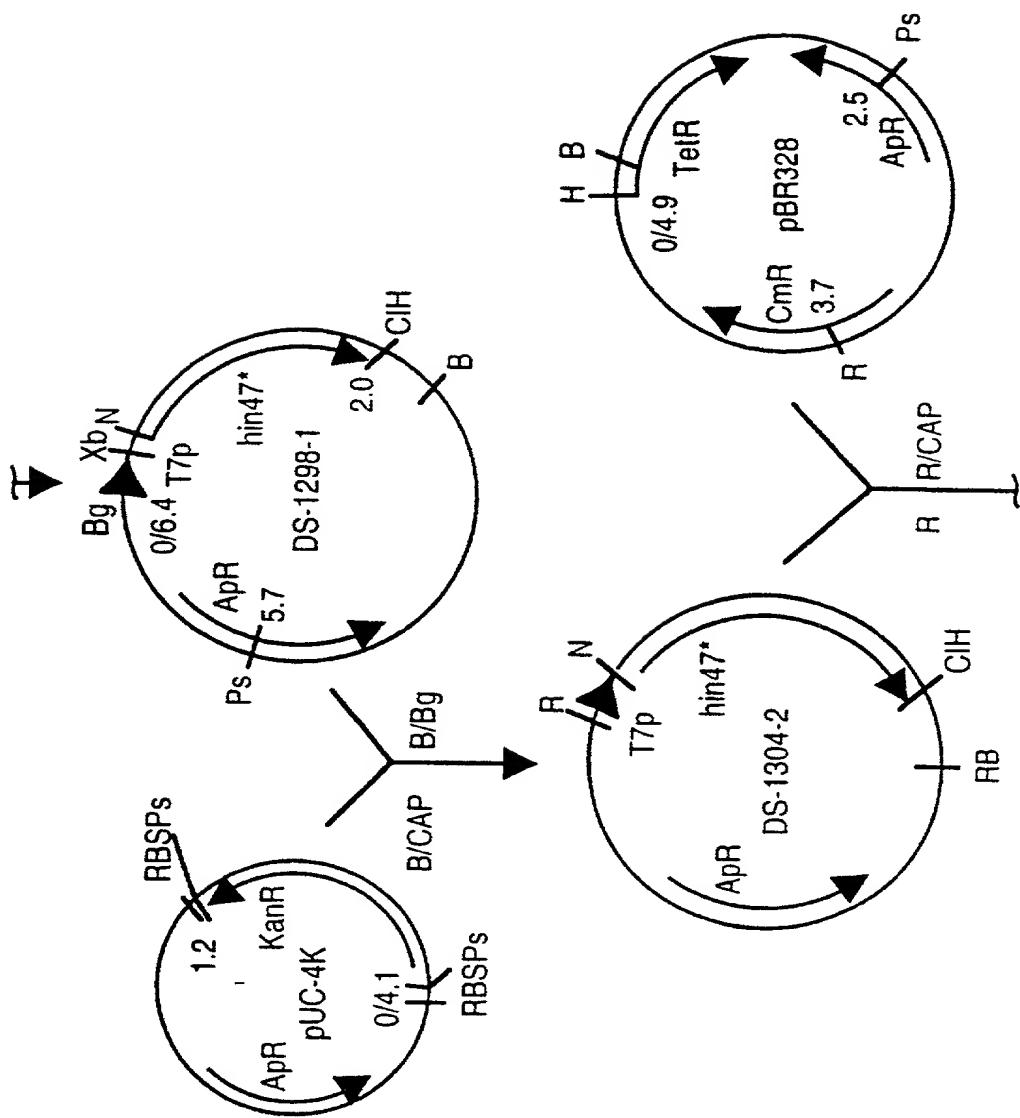


FIG.8B

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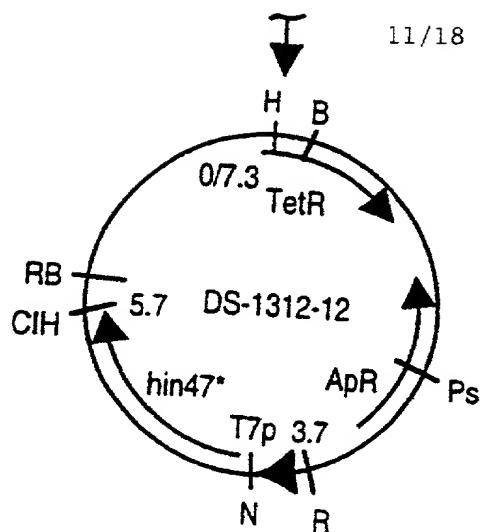
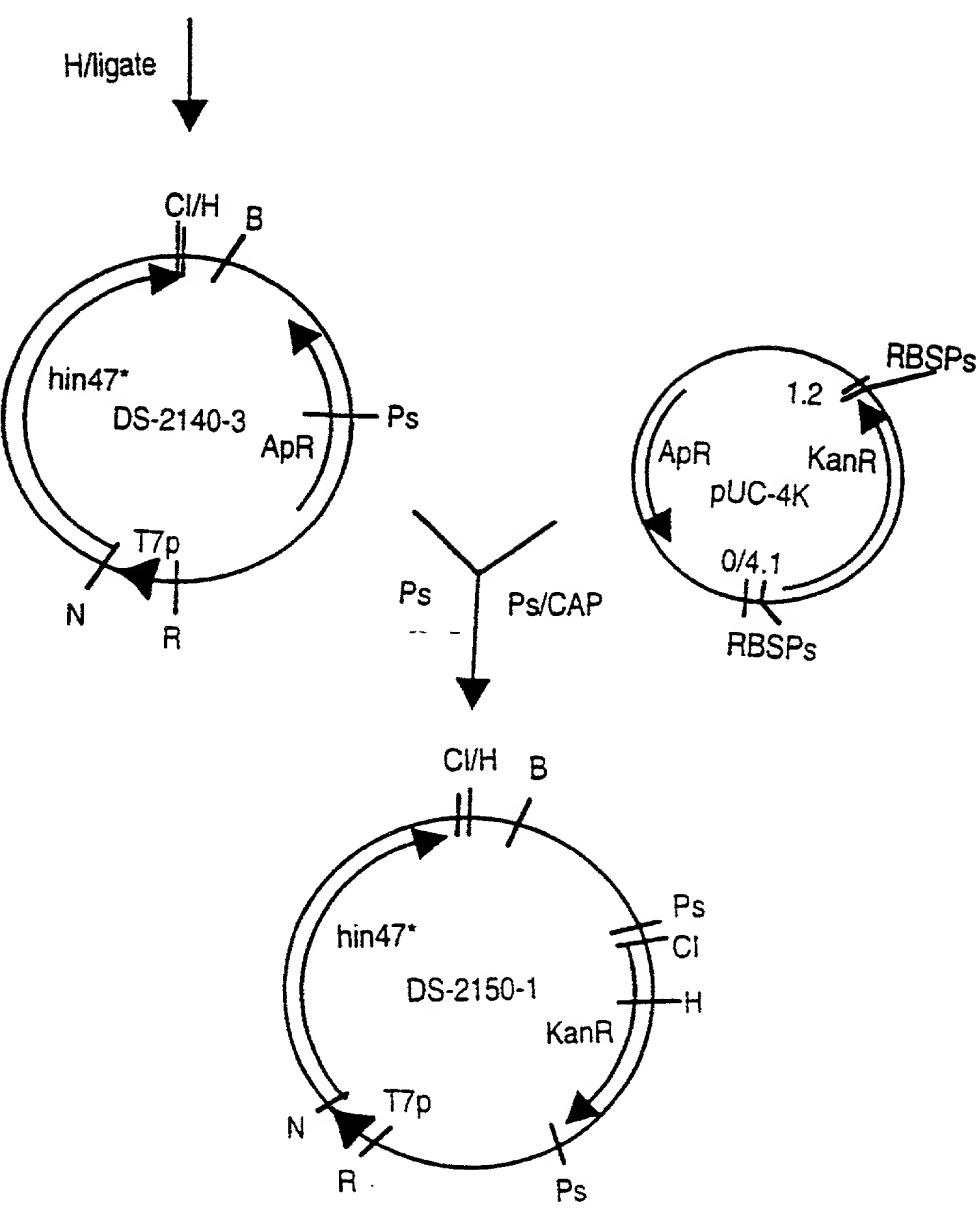


FIG.8C



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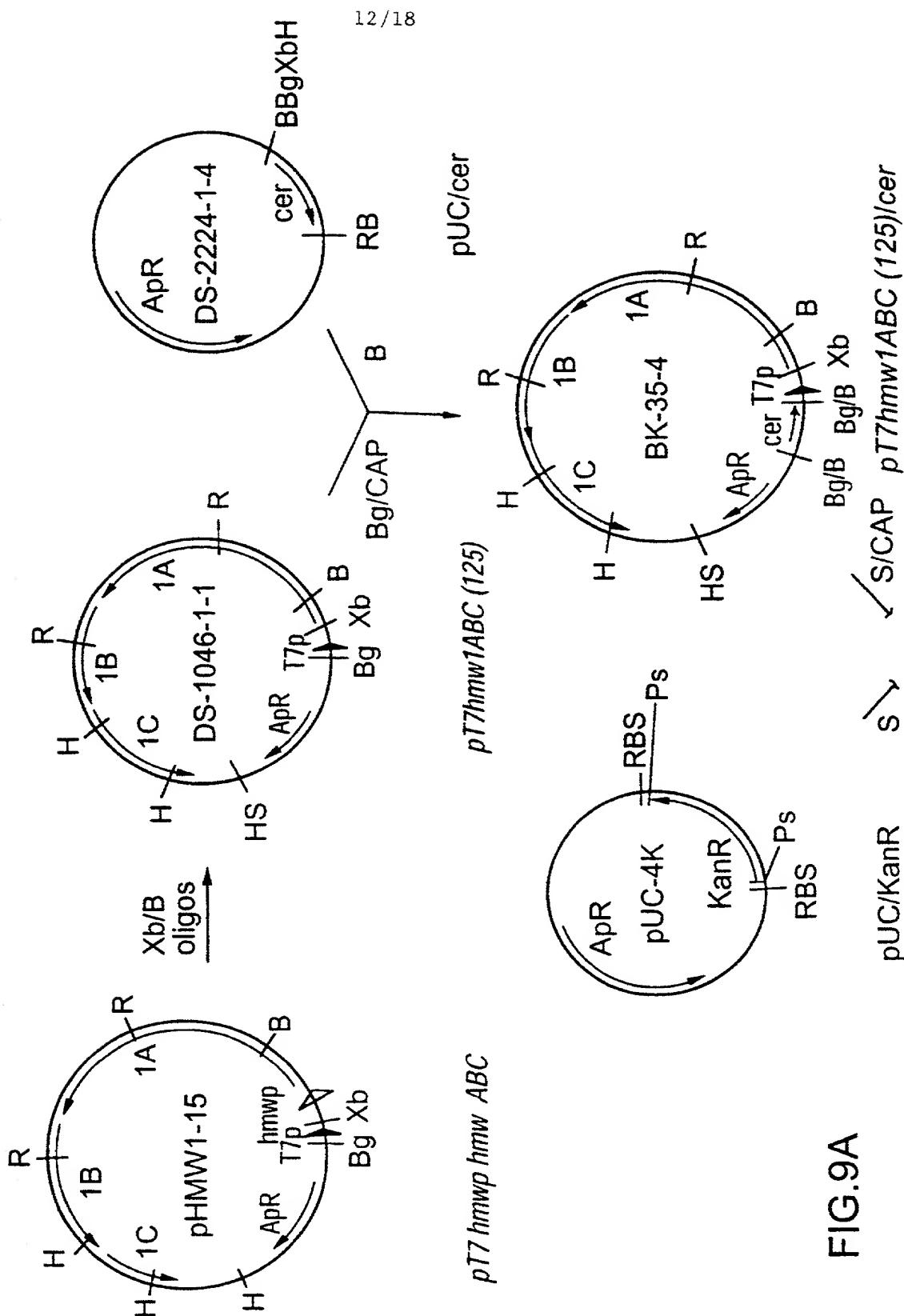
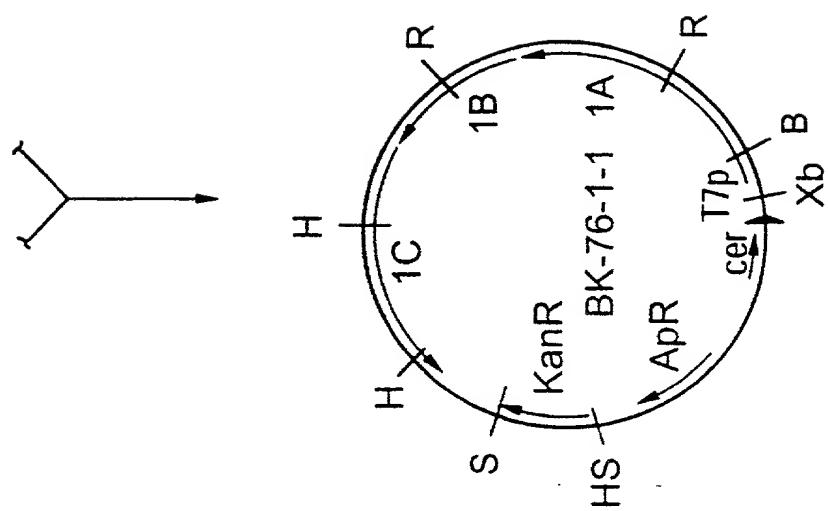


FIG.9A

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*pT7hmw1ABC (125)/cer/KanR*

**FIG. 9B**

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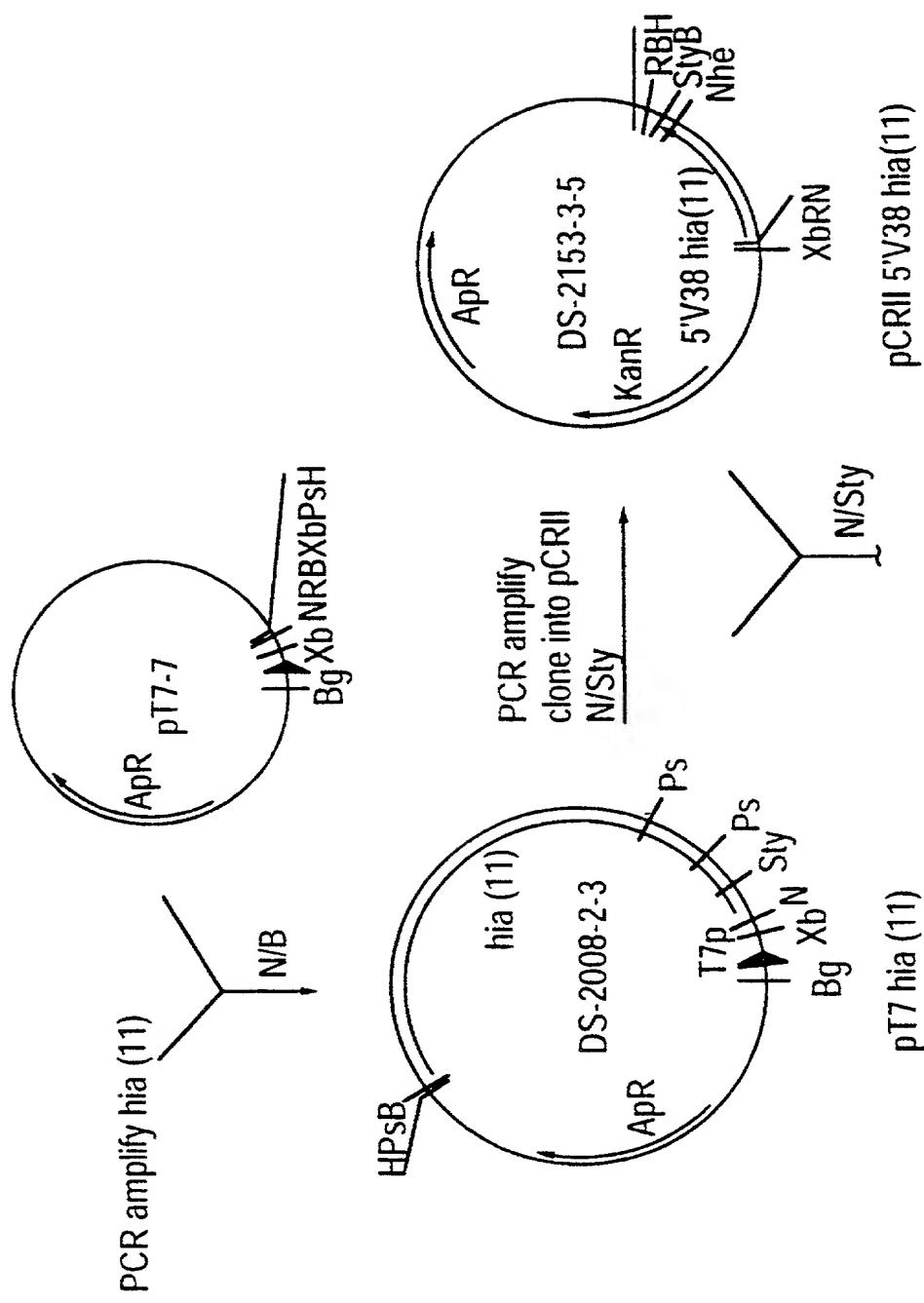


FIG. 10A

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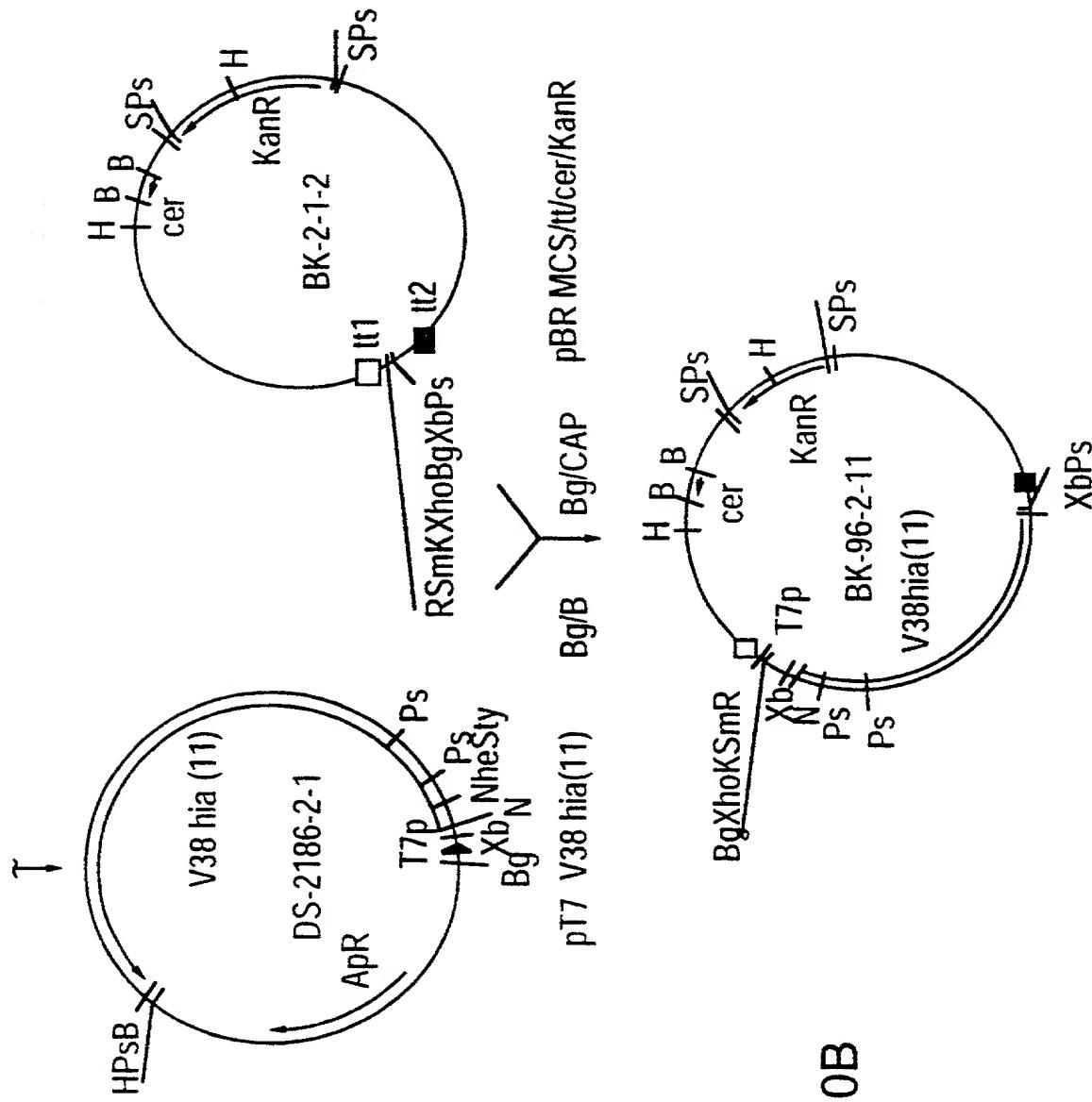


FIG. 10B

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Protective Ability of H91A Against Intrabulla Challenge  
with NTHi Strain 12 in Chinchillas

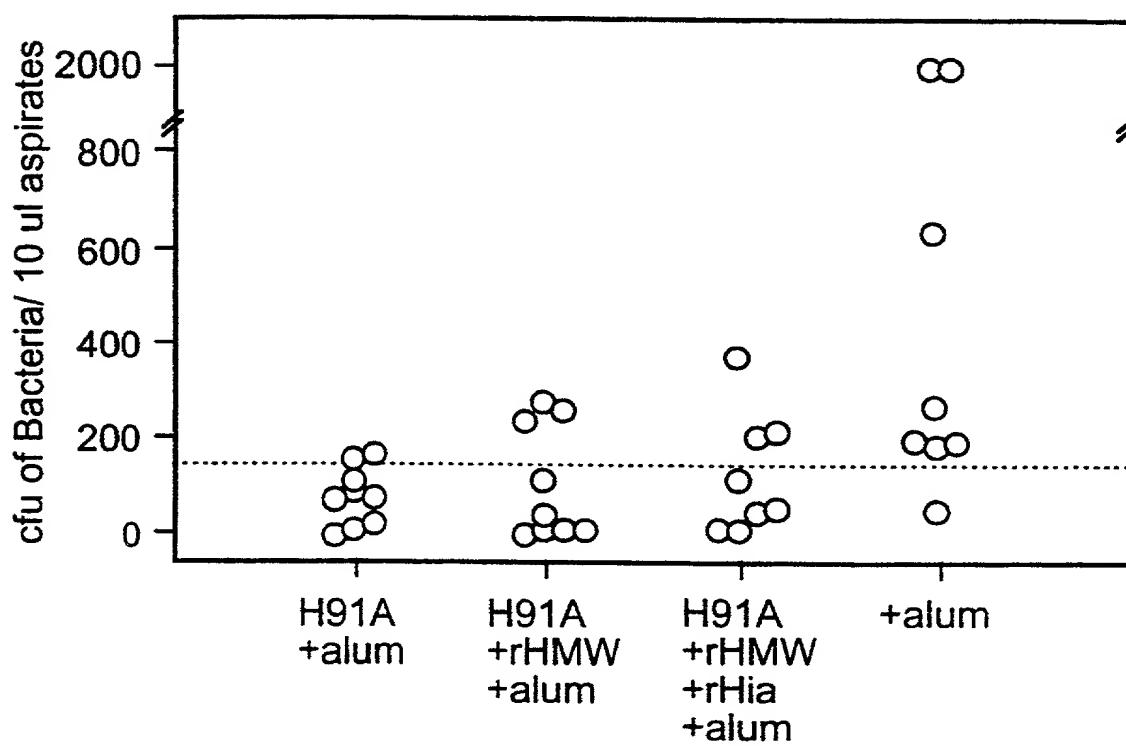


FIG.13

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Oligonucleotides used to PCR amplify the full-length strain 11 *hia* gene for expression studies.

sense

EcoR I Nde I  
↓  
↓

5' GCGAATTCAATGAAACAATTITTAACGTATTGGAAAT 3'

antisense

5' K T G V A A G V G Y Q W \* \*  
AAAATAGGGTTTGCAGCAGTTGTTACCACTGGTAATAG  
3'  
3' TTTCGTCGCCAACGTTGCTAACCAATGGTACCCATTATCTTAAGGCCTAGGGCG  
5'  
EcoR I BamH I  
↑  
↑

SEQ ID no: 2  
SEQ ID no: 3

5038.SL  
5039.SL

SEQ ID no: 4  
SEQ ID no: 5  
SEQ ID no: 6

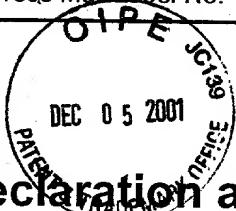
FIG. 11

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Oligonucleotides primers to PCR amplify truncated V38 strain 11 hia gene



FIG.12



Docket No.  
1038-1183MIS:jb

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

### MULTI-COMPONENT VACCINE COMPRISING AT LEAST THREE ANTIGENS TO PROTECT AGAINST DISEASE CAUSED BY HAEMOPHILUS INFLUENZAE

the specification of which  
(check one)

is attached hereto.

was filed on February 29, 2000 as United States Application No. or PCT International Application Number PCT/CA00/00207  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

#### Prior Foreign Application(s)

Priority Not Claimed

(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

---

(Application Serial No.)

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(Filing Date)

---

(Application Serial No.)

---

(Filing Date)

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(Application Serial No.)

---

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

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**09/261,182**

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**March 3, 1999**

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**Pending**

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(Application Serial No.)

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(Filing Date)

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(Status)

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(patented, pending, abandoned)

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**PCT/CA00/00207**

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**February 29, 2000**

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(Status)

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(patented, pending, abandoned)

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(Application Serial No.)

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(Filing Date)

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(Status)

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(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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Third inventor's signature

Date

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*Nov. 13, 2001*

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Fourth inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of fifth inventor, if any

Fifth inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of sixth inventor, if any

Sixth inventor's signature

Date

Residence

Citizenship

Post Office Address